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14. ABSTRACT
The purpose of this project is to evaluate subjects with Gulf War illness (GWI) for pathogens and potential biomarkers. The scope of this work will primarily encompass transcriptional analysis of lymphocytes from individual with GWI and compare the results to the same data from healthy control volunteers. Any potential pathogens identified will be confirmed by complimentary methods. Additionally, we will also survey the production of inflammatory cytokines and chemokines from the same individuals as well as evaluate lymphocyte populations. This information will be used to produce a diagnostic signature that can be used to delineate those who suffer from GWI from those who do not. At this time, we are developing a potential diagnostic signature that can be used to distinguish GWI from healthy controls and a closely related illness, chronic fatigue syndrome (ME/CFS).

15. SUBJECT TERMS
GWI, cytokines, next generation sequencing

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1. INTRODUCTION:

The aim of this project was to evaluate subjects with Gulf War illness (GWI) for pathogens and potential biomarkers. In order to fulfill this mandate, we have focused on evaluating the transcriptome of circulating lymphocytes as well as searching for any potential blood-associated pathogens. The transcriptome is reflective of the genes that are being actively expressed at any given time; therefore, the lymphocyte transcriptome represents a window into the immune system, potentially leading to an understanding of GWI pathogenesis. Transcriptome analysis also has the ability to identify any pathogens present in the immune cells or circulating in the blood, thus, potentially identifying an etiological trigger. In addition, we also evaluated serum cytokines and chemokines and use this data to identify a potential diagnostic signature. By identifying potential biomarkers including pathogens associated with GWI, the results of this study may afford physicians the necessary tools to make more accurate diagnoses and improve subject care. At the time we closed study recruitment we had consented 42 subjects with GWI and 32 control subjects. GWI cases comprised of veterans who were on active duty during the Gulf War era (Desert Storm: 1990-1991) and have symptoms of GWI. Control subjects were individual volunteers who have no symptoms of GWI and are deemed healthy at the time of their involvment.

2. KEY WORDS:

Gulf war illness; cytokines; chemokines; RNAseq; Th1; Th17

3. OVERALL PROJECT SUMMARY

Change in original SOW. The original proposed aim of this study was to establish the prevalence of a newly identified infectious retrovirus in individuals with GWI. The original Principal Investigator (PI) left the Whittemore Peterson Institute ("WPI") shortly after this study was funded. Therefore, a petition was made to have the PI changed to Dr. Vincent Lombardi. In light of several subsequent research reports that questioned the possibility of this newly identified retrovirus as a human pathogen, Dr. Lombardi also made a request to the Army Contracting Officer Representative to amend the original proposal to broaden the scope of the pathogen discovery aspect of this study by utilizing next generation sequencing (NGS) technology to allow any pathogen to be identified, including, but not limited to, the originally proposed retrovirus. Additionally, this technology has the potential to identify useful biomarkers and immune dysregulation through transcriptome analysis, which was not addressed in the original proposal, but was incorporated into the amended proposal.

Upon receiving approval to implement the requested modifications to the original proposal, a new human subjects protocol was required. The WPI and the VA Sierra Nevada Health Care System ("VASNHCS") worked together to define the study population and prepare and submit the protocol, including all consent forms and recruitment materials. The subject protocol was approved by the University of Nevada Reno (UNR) Institutional Review Board (IRB) on June 26, 2012, and by the VA Sierra Nevada Health Care System (VASNHCS) Research and Development Department (R&D) on July, 26 2012. The subject protocol was also reviewed by the U.S. Army Medical Research and Materiel Command (USAMRMC), Office of Research Protections (ORP), Human Research Protection Office (HRPO) and found to comply with applicable DOD, U.S. Army, and USAMRMC human subjects protection requirement on August 1, 2012. Additionally, a modification to the IRB allowing for the use of new recruiting documents was approved on September 25, 2012. This completed Task 1a as outlined in the Statement of Work (SOW) for months 0-6 as predicted in the proposed timeline.

After approval of the protocol, the VASNHCS commenced recruiting operations. This process was continued until a total of 42 cases and 32 controls were consented into the study. The recruitment was less than we had anticipated however; we were limited to the availability of study subjects

recruited by the local VA. The completion or recruitment of study subjects and collection of biological specimens completed Task 1b and Task 2 of the SOW.

The mandate of Task 3 was the completion of transcriptome analysis by next generation sequencing. Our initial plan was to sort cryopreserved lymphocyte populations and then conduct transcriptional analysis on sorted B cells, T cells, Natural Killer (NK) cells, Monocytes and plasmacytoid dendritic cells (pDCs). However, our quality control evaluation determined that the integrity of the RNA was not consistent, likely due to the time delay in sorting each population. The collection of specimens included whole blood, from which lymphocytes were isolated, serum, plasma and RNA from whole blood collected in RNA preservation tubes. Therefore, we elected to conduct transcriptome profiling of whole blood. The sequencing was being conducted right up to the end of the study and is completed as of this date. However, we are conducting additional data analysis and anticipate a manuscript of this data will be submitted before the end of the year. A first draft of this manuscript is attached and the summary of the preliminary findings are give below.

Cytokine Analysis

Previous studies have reported that inflammatory cytokines are differentially expressed in individuals with GWI and therefore, cytokine expression may yield a signature that can serve as a useful biomarker. To explore this possibility, we took a two-perspective approach. Our first approach was to analyze the kinetics of cytokine production of PBMCs in response to the toll-like receptor (TLR)-4 agonists LPS. Our preliminary data suggested that GWI subjects might have a pDC dysfunction; therefore, we also measured the response of pDCs to the TLR-7 agonist ODN and the TLR-9 agonist Imiquimod. Our second approach was to conduct the most comprehensive cytokine survey to date on subjects with GWI and compare these data to healthy controls and controls with ME. We originally intended to publish this work as a comprehensive manuscript; however, we elected to split this work into two manuscripts. The manuscript titled “Cytokine expression provides clues to the pathophysiology of Gulf War illness and myalgic encephalomyelitis” was submitted to the journal Cytokine and was accepted on October 31st with minor revisions. The revised manuscript was submitted and accepted on November 20th; proofs have been sent and we are now waiting for the publication date. The accepted manuscript is attached. The second manuscript titled “Inflammatory cytokine kinetics differentiates Gulf War Syndrome and Myalgic encephalomyelitis” is currently being prepared and we expect to have this manuscript submitted before the end of January. Once accepted, we will forward the manuscript to the program officer. A summary of both studies and the results of each study are presented below.

Cytokine expression provides clues to the pathophysiology of Gulf War illness and myalgic encephalomyelitis. In this study**,** we measured 77 serum cytokines in subjects with GWI and compared these data to healthy controls. Additionally, because a useful biomarker must delineate the subjects of interest from those with similar symptoms, we also compared these data to that of subjects with myalgic encephalomyelitis (ME), also known as chronic fatigue syndrome, a disease with similar and overlapping symptomology. In fact, it has been suggested that all GWI cases meet the diagnostic criteria for ME and, therefore, represent discrete subsets of ME; however, the results of this study suggest that GWI is not a subset of ME.

This study included a total of 146 subjects; 67 cases with a confirmed diagnosis of ME, 37 identified as having GWI, and 42 healthy controls. Serum cytokine levels were analyzed on a Luminex 200 analyzer with Bio-Plex (Bio-Rad) multiplex magnetic bead-based antibody detection kits. In order to determine differences in cytokine values and distributions between GWI, ME, and control subjects, we initially performed Kolmogorov-Smirnov tests for normality, which revealed that the data were not normally distributed (data not shown). We therefore used the non-parametric Kruskal–Wallis (K.W.) one-way analysis of variance by ranks to confirm that the three populations did not originate from the

same distribution. We then performed a Mann-Whittney (M.W.) analysis to identify differences in medians between GWI and ME cases as well as between GWI cases and controls and ME cases and controls. We additionally conducted Pearson correlation analysis, comparing cytokines to each other within each respective subject population. Finally, we performed classification analysis using the tree-based ensemble machine learning algorithm Random Forest [1]. For this analysis, 500 random trees were built using six predictors for each node, and auto-bootstrap out-of-bag sampling was used for testing the model.

Of the 77 cytokines analyzed, 48 (63%) differed for at least one of the three groups ($p \leq 0.05$ by Kruskal–Wallis), suggesting that the respective cytokine values did not originate from the same distribution (Supplemental data of manuscript Table 1). We next utilized the Mann-Whittney test to compare the two groups of cases with each other and each group of cases with the control group (Supplemental data of manuscript Table 1). When GWI and ME subjects were compared, 48 cytokines were observed to be significantly different ($p \leq 0.05$). Additionally, when ME cases were compared to healthy controls, 42 cytokines were observed to be differentially expressed, 17 of which were upregulated and 26 were downregulated (Supplemental data of manuscript Table 2). This is in contrast to only 14 cytokines that differed between GWI cases and controls, 7 of which were upregulated and 7 were downregulated (Table 1.).

Previous studies have reported that Th1, Th2, and Th17 cytokines, or combinations thereof, characterize GWI and ME [2-6]. To explore this possibility, we organized these cytokines into three groups (Table 2). For subjects with GWI, the Th1 cytokine IFN-γ was upregulated; however, CXCL8 was slightly downregulated. Additionally, the Th2 cytokines IL-4, IL-13 and IL-25 were all significantly downregulated. For the Th17 cytokines, we observed IL-17F to be significantly downregulated in ME cases, while IL17A and IL17F were both significantly upregulated in GWI cases. Of the 48 cytokines that were differentially expressed in ME, 12 (25%) represented Th1, Th2, or Th17 cytokines. In contrast, of the 14 differentially expressed cytokines observed in GWI subjects (Table 2), 9 (64%) represented Th1, Th2, or Th17 cytokines.

Table 1. Cytokines upregulated and downregulated in subjects with Gulf War illness (GWI) when compared to healthy controls

Cytokines upregulated in GWI subjects

Analyte	Group	Minimum	Maximum	Mean	Standard Deviation	P value by M.W.
CCL11	CON	20.7	90.9	47.1	16.4	
	GWI	22.2	252.8	72	48.4	0.008
FGF	CON	3	98.5	20.7	17	
	GWI	9.2	57.7	23.9	11.1	0.026
IFN-γ	CON	2.8	50.4	15.4	10.3	
	GWI	7	47.5	22.4	10.6	0.003
IL-17A	CON	2.5	10.5	7.5	1.5	
	GWI	0.8	277.8	13.6	44.8	0.032
IL-17F	CON	1	143.5	17.9	30.8	
	GWI	9.4	678.8	47.8	113.5	0.001
IL-33	CON	8.2	2850.6	723.3	646	
	GWI	56.5	4379.6	1112.2	883.4	0.011
IL-5	CON	2.4	6.4	5.9	0.9	
	GWI	0.1	24.5	6.4	3.4	0.014

Cytokines downregulated in GWI subjects

Analyte	Group	Minimum	Maximum	Mean	Standard Deviation	P value by M.W.
CCL5	CON	300.4	27934.9	5520.3	4290.5	
	GWI	497.4	10415.4	4059.3	2015	0.031
CXCL8	CON	6.4	3660.2	267.4	620.8	
	GWI	2.2	1413.5	126.3	284	0.032
IL-13	CON	8.1	11.3	9	1.3	
	GWI	8.1	13.9	8.2	1	0.001
IL-25	CON	0	4.7	2.4	2	
	GWI	0	4.7	1.3	1.4	0.04
IL-4	CON	0.1	2	1.1	0.6	
	GWI	0.1	4.6	0.9	0.7	0.041
IL-7	CON	1.4	11.8	5.5	3.2	
	GWI	2.5	18.9	3.7	2.7	0.011
TNF-α	CON	17.6	72.2	23.1	10.2	
	GWI	5.7	20	19.3	3	<0.0001

Table 2. Th1, Th2, and Th17 cytokines expression in subjects with GWI and ME

Analyte	**CON**	**ME**	**GWI**	**P value by Mann Whittney ME vs CON**	**GWI vs CON**
Th1 Cytokines					
IFN-γ	15.4	32.4	22.4	0.001	0.003
IL-2	4.7	11.3	3.6	0.062	0.271
TNF-α	23.1	45.4	19.3	<0.0001	<0.0001
IL-12(p75)	20	32.7	21.9	<0.0001	0.246
CXCL8	267.4	191.9	126.3	<0.0001	0.032
IL-18	790.2	726.3	873.2	0.219	0.446
IL-12(p40)	290.1	238.3	309.9	0.089	0.914
Th2 cytokines					
IL-4	1.1	1.7	0.9	<0.0001	0.041
IL-13	9	10.7	8.2	<0.0001	0.001
IL-1β	7.9	14.4	6.6	<0.0001	0.133
IL-25	2.4	5	1.3	<0.0001	0.04
IL-10	10.2	31.7	10.8	0.017	0.223
IL-5	5.9	5.3	6.4	<0.0001	0.014
IL-6	4.3	12.4	3.6	<0.0001	0.202
IL-9	31.7	8	8.7	<0.0001	0.1
Th17 cytokines					
IL-17A	7.5	8.7	13.6	0.053	0.032
IL-17F	17.9	2.9	47.8	<0.0001	0.001
IL-21	30.1	44.5	36.2	0.918	0.081

In addition to T cells, other cells make many of the cytokines typically associated with a Th1, Th2 or Th17 shifts. For instance, the endogenous pyrogen TNF-α is primarily made by activated macrophages, but is also made by most nucleated cells including lymphocytes, fibroblasts and

neurons [7, 8]. Likewise, IL-6 is produced by activated macrophages as well as T cells and can act in a proinflammatory or antiinflammatory capacity [9]. Therefore, we conducted correlation analysis, in order to provide additional clues as to which cells produce these cytokines in our study groups. Our results suggest the Th1 cytokines strongly correlate in the ME population but substantially less so for the Th2 cytokines (Table 3.). Additionally, a complete absence of correlation was observed for the Th17 cytokines in the ME group. In contrast to the ME group, only IL-2 and IL-12(p75) showed any significant correlation in GWI. Again, with respect to the GWI group, the Th1 cytokines showed a much weaker correlation and strikingly, TNF-α was negatively correlated with IFN-γ and IL-2, which is in contrast to the ME and control groups (Table 3.). Also, the Th17 cytokines showed a moderately positive correlation in the GWI population. Interestingly, the correlation between IL-17A and IL-21 showed a positive correlation in contrast to that of the control population, which showed a negative correlation.

In addition to the Th1, Th2 and Th17 cytokines, we conducted correlation analysis on the remaining analytes and observed almost perfect correlation (R2 ≥0.90) between a number of cytokines in the ME group (26 cytokines) but fewer in the GWI group (14 cytokines) (Supplemental data Table 3). Of particular interest, we observed almost perfect correlation between IL-7 / IL-13, IL-7 / FGF, and IL-7 / TNF-α and between IL-3 / IFN-α in the ME group. We also observed near perfect correlation between IL-1α / IL-3, IL-1α / IL-12(p40), IL-1α / IL-2 and IL-1α / IL-22 and between IL-1 / IL-7 and IL-1 / IL-13 in the GWI group. Both GWI and ME are diseases with undefined etiology and both are often characterized by aberrant cytokine expression; however, the patterns of cytokine expression appear to be more complex than can be described by a standard Th1, Th2 or Th17 shift. With this in mind, we utilized the machine logic algorithm Random Forest (RF) to analyze our data set and potentially identify the most important cytokines that define these diseases. For this analysis, 500 random decision trees were built using six predictors for each node, and auto bootstrap out-of-bag sampling was employed to test the model. The 20 most significant cytokines for delineation of each group of subjects in order of decreasing importance were IL-7, IL-4, TNF-α, IL-13, IL-17F, IL-1, IL-5, IL-25, CXCL8, VEGF, CCL11, IL12(p75), IL-9, CFS3, IFN-γ, CCL4, IL-6, CCL2, CXCL1, and CXCL10 (Figure 1.). Using only serum cytokines, we were able to achieve sensitivity of 92.5% for delineating ME; however, only 64.9% sensitivity was achieved when delineating GWI with 33.3% overall specificity (Table 3). These data indicate that using serum cytokines alone may not yield an effective diagnostic tool; however, it may provide important clues regarding the underlying pathology of the disease.

Table 3. Correlation of cytokines in ME, GWI and controls (values given as R-squared)

Th1 Cytokines ME				Th1 Cytokines GWI				Th1 Cytokines CON			
	IL-12p75	TNFα	IL-2		IL-12p75	TNFα	IL-2		IL-12p75	TNFα	IL-2
IFNγ	0.944	0.797	0.934	IFNγ	0.408	-0.495	0.424	IFNγ	0.305	0.346	0.339
IL-2	0.929	0.562		IL-2	0.724	-0.166		IL-2	0.837	0.802	
TNFα	0.758			TNFα	0.012			TNFα	0.534		

Th2 Cytokines ME					Th2 Cytokines GWI					Th2 Cytokines CON				
	IL-9	IL-6	IL-5	IL-13		IL-9	IL-6	IL-5	IL-13		IL-9	IL-6	IL-5	IL-13
IL-4	0.065	0.282	0.561	0.679	IL-4	0.354	0.152	0.674	0.872	IL-4	-0.011	0.660	-0.742	0.878
IL-13	0.074	0.068	0.946		IL-13	0.249	0.16	0.896		IL-13	-0.121	0.680	-0.633	
IL-5	0.056	0.019			IL-5	0.306	0.117			IL-5	0.113	-0.462		
IL-6	-0.017				IL-6	0.013				IL-6	0.041			

Th17 Cytokines ME			Th17 Cytokines GWI			Th17 Cytokines CON		
	IL-21	IL-17F		IL-21	IL-17F		IL-21	IL-17F
IL-17A	-0.016	-0.022	IL-17A	0.349	0.102	IL-17A	-0.544	-0.337

IL-17F	-0.036	IL-17F	0.592	IL-17F	0.815

FIGURES

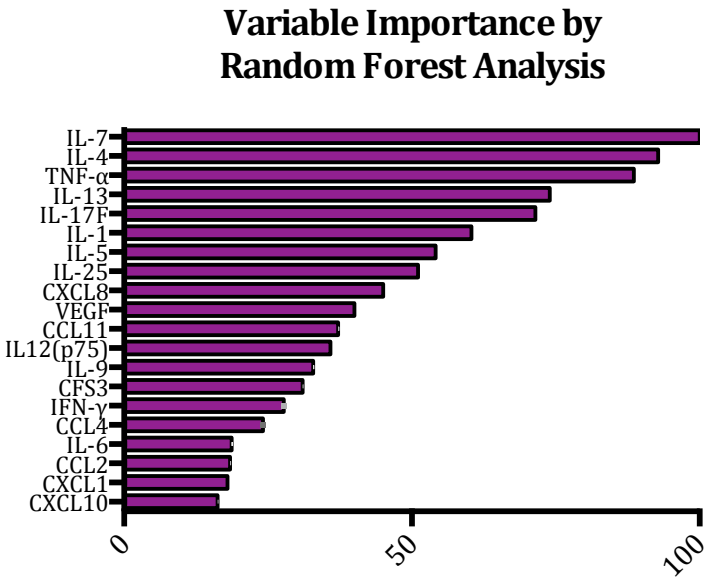

Figure 1. Classification analysis of cytokine data using Random Forest. In order to identify which cytokines most accurately predict disease status of subjects with GWI, ME or controls, Random Forest analysis was implemented whereby 500 random trees were built and six predictors were used at each node. Auto-bootstrap out-of-bag sampling was used for testing the model.

Table 4. Random Forest model statistics

Out-of-Bag Testing Misclassification				
Class	N Cases	N Misclassified	Pct. Error	Cost
ME	67	5	7.46%	0.0746
GWI	37	13	35.14%	0.3514
CON	42	28	66.67%	0.6667

Out-of-Bag Testing Prediction Success					
Actual Class	Total Class	Percent Correct	ME N = 70	GWI N = 46	CON N = 30
ME	67	92.54%	62	1	4
GWI	37	64.86%	1	24	12
CON	42	33.33%	7	21	14
Total:	146				
Average:		63.58%			
Overall% Correct:		68.49%			

Summary points of this study

1. This study suggests that GWI and ME are distinct disease
2. This study supports a Th1/TH17 shift in GWI
3. This study suggests that a ratio of IL-17A and IL-21 can discriminate GWI, ME and healthy controls
4. This study identifies IL-7, IL-4, TNF-α, IL-13, IL-17F, IL-1 as the most important cytokines that contribute to GWI pathology.
5. This study identified 14 cytokines that are differentially expressed in subjects with GWI.

The accepted manuscript of this work is attached hereto.

Inflammatory cytokine kinetics differentiates Gulf War Syndrome and myalgic encephalomyelitis. Toll like receptors (TLRs) are pattern recognition receptors that are activated by molecular motifs shared by pathogens, but that are typically not present in the host. There are several well-defined TLRs with specific activating ligands. For example, TLR4 is activated by the bacterial product lipopolysacharide (LPS) while single stranded RNA and bacterial DNA, typically of viral origin; activate TLR 7 and 9, respectively. Activation of various TLRs can trigger the expression of a unique pattern of cytokines, influencing disease progression and outcome. As part of our investigation into the control of inflammatory cytokines, we sought to determine the effect upon stimulation of various TLRs expressed by PBMC and plasmacytoid dendritic cells from GWI cases and control donors. The TLR4 agonist, LPS and TLR7 and 9 agonists Imiquimod (IMQ) and ODN 2216 (ODN) [10], respectively, were used to study the inflammatory kinetics associated with GWI. TNFα was chosen to follow TLR4 stimulation and IFNα was used to follow the stimulation of TLR 7 and 9. Our rational for following these TLRs was based upon our preliminary cytokine screening that suggested that IL-7 and TNFα were dysregulated in GWI. IL-7 is primarily made in the bone marrow but is only made in the periphery by dendritic cells and TNFα is made in response to most TLRs.

In this study, peripheral blood mononuclear cells (PBMCs) ($2x10^5$) from GWI cases and controls were stimulated with LPS (10 ng/ml and 1µg/ml) [11] directly after seeding culture plates, followed by stimulation at 16, 24, 32, 38, and 48 hours. Culture medium was harvested at 2, 4, 6, 8, 10, 12, 14, 16, 18, 24, 26, 32, 34, 38, 40, 48, and 50 hours after LPS stimulation and stored at -80 °C until assayed. Culture medium was replaced each time with fresh medium. PBMC stimulation response is presented in Figure 2. pDCs were isolated using Miltenyi CD304 (BDCA-4/Neuropilin) microbead kit and were allowed to rest for 18 hours after isolation before adding stimulants. pDCs were stimulated with IMQ (5 ug/mL) or ODN (5 uM). IMQ and ODN stimulation response and culture medium harvesting was performed similar to experiments with LPS stimulation of PBMC. Concentration of TNFα and IFNα were determined by microplate ELISA.

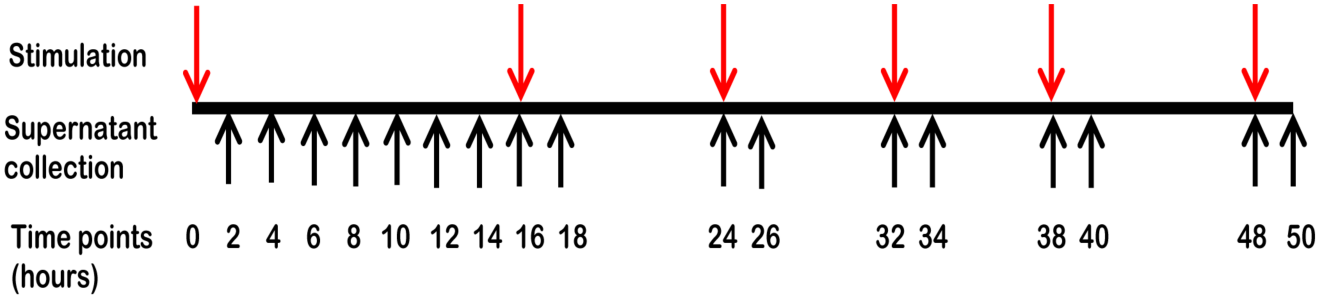

Fig.2 PBMC stimulation experiment; mode of stimulation and supernatant collection.

LPS (10 ng/ml) was added to the PBMC cultures at 0, 16, 24, 32, 38, and 48 hours. Concentration of TNFα in supernatants was determined at selected time points. With exception of 24 hours post stimulation, TNFα concentration did not differ significantly in supernatants of PBMC collected from GWI cases and controls (Fig 3). At 24 hours, TNFα concentration in the supernatant of PBMC from GWI cases was significantly higher compared to that in controls. Our data suggests that PBMC from GWI subjects maintain ability to activate TNFα when stimulated with low concentration of LPS similar to that in controls but produce slightly more TNFα at 24 hrs.

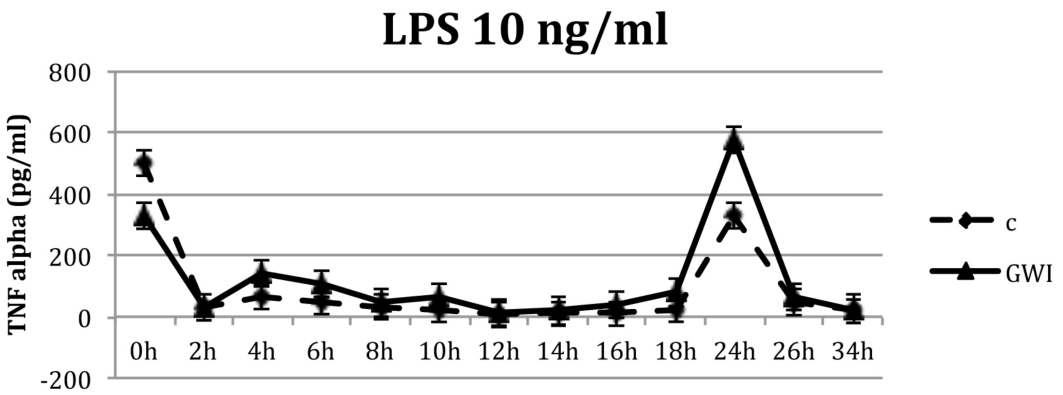

Fig. 3. TNF-α activation in PBMC from GWI subjects and healthy controls stimulated with LPS (10 ng/mL).

We then investigated the effect of a higher concentration of LPS (1 μg/ml) on TNFα activation in PBMC from GWI cases and controls. The mode of LPS stimulation and supernatant collection is the same as described in Fig 2. A higher concentration of LPS (1 μg/ml) produced a similar pattern of TNFα activation as that produced by low concentration of TNFα (10 ng/ml) (Fig 4) with the exception that this time, GWI subjects produced slightly less TNFα at the 24-hour time point. Since LPS acts through activation of TLR4, we conclude that PBMC from GWI cases retains ability to respond to TLR4 agonists similar to that of healthy donors at high concentrations, but GWI cases are more sensitive to low level LPS stimulation. These data suggest that GWI cases may respond to low-level bacterial-induced inflammation to a greater extent than control subjects.

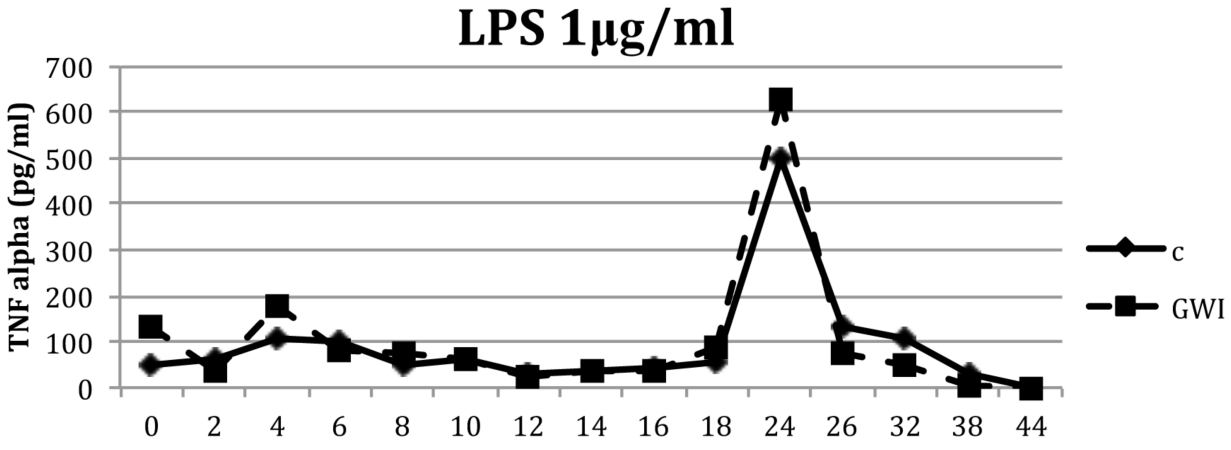

Fig 4. TNFα activation in PBMC from GWI subjects and healthy controls stimulated with LPS (1μg/ml).

Next, we sought to determine the effect of ODN and IMQ on the expression of IFNα by pDCs from GWI subjects and healthy controls. Cell culture supernatants were collected at selected time points and used to determine IFNα concentration by ELISA assay.

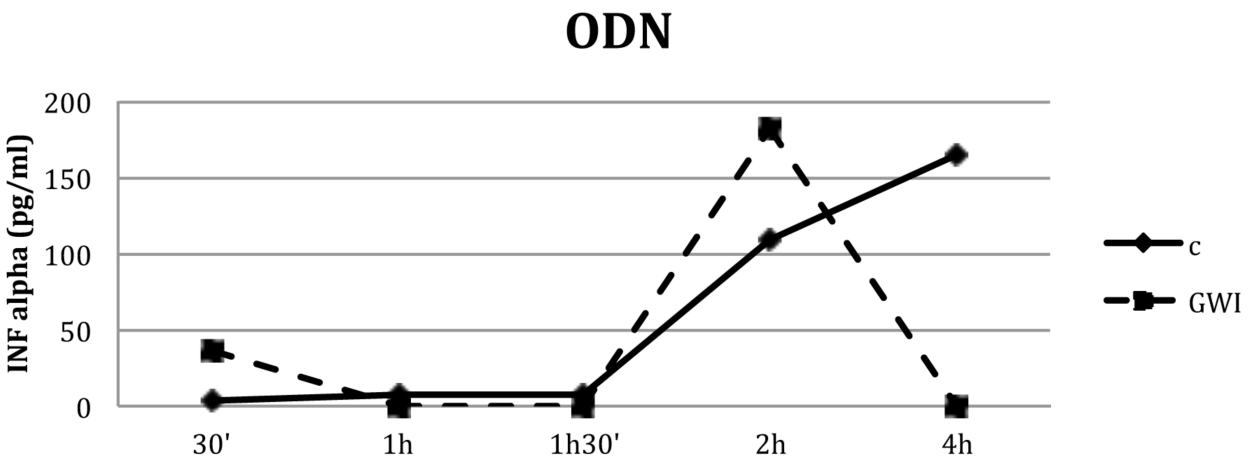

Fig. 5. Effect of ODN stimulation on IFNα production by pDCs from GWI subjects and healthy subjects.

The concentration of IFNα in the supernatants from control pDCs cultures was increased at 2 and 4 hours after stimulation with ODN (Fig 5). The concentration of IFNα in the supernatants from GWI pDC cultures increased similar to that in controls by 2 hours after stimulation. However, levels of IFNα declined, returning to initial levels by 4 hours post stimulation with ODN suggesting a marked difference between cases and controls. Upon treatment of control pDCs with IMQ, IFNα expression increased to a maximum at 1-hour post stimulation and declined thereafter. In contrast, IFNα production by pDCs of GWI cases stimulated with IMQ was negligible (Fig 6). Repeating this experiment twice produced similar results.

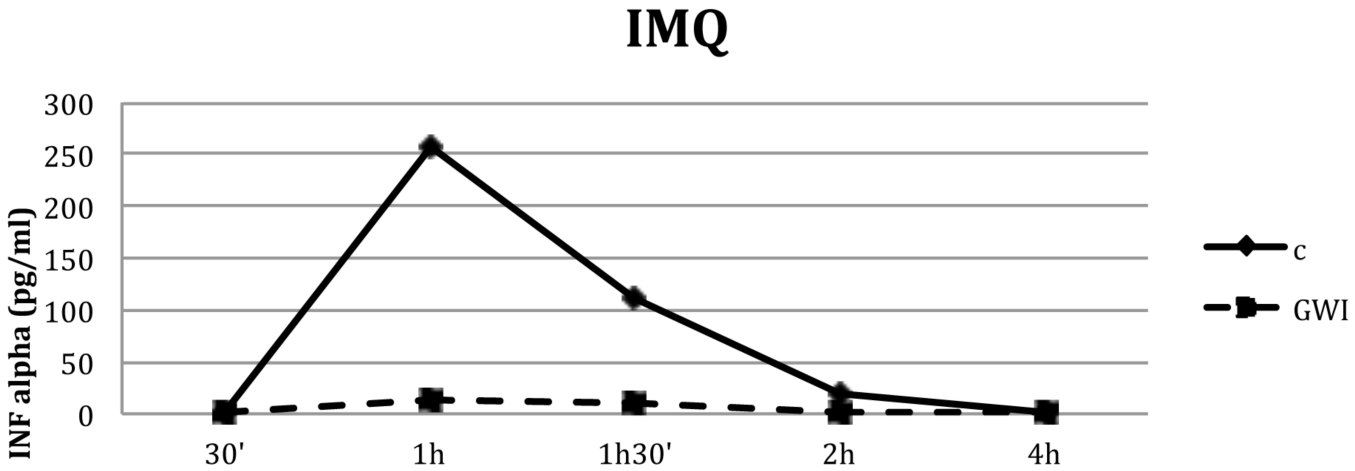

Fig. 6 Effect of IMQ on IFNα activation in pDC from GWI subjects.

Summary points of this study

1. This study suggests that immune cells from GWI subjects are hyper-responsive to the lipopolysaccharides produced by Gram-negative bacteria.
2. This study suggests that plasmacytoid dendritic cells are dysregulated in subjects with GWI, potentially at the level of TLR-7.

Transcriptome analysis

In order to explore the underlying pathophysiology of GWI and potentially identify differentially expressed genes, we conducted whole blood transcriptome analysis of cases and controls by next-generation RNA sequencing (RNA-Seq). We then implemented the biological pathway analysis to identify differentially expressed genes common to biochemical pathways. Our results primarily implicate mitochondrial-related proteins in the pathophysiology of GWI and thus support previous clinical studies that suggest mitochondrial dysfunction may contribute to the pathophysiology of GWI. This report, to the best of our knowledge, is the first to link specific mitochondrial-related proteins in association with GWI.

Transcriptome analysis identifies relevant pathways that may contribute to the pathophysiology of Gulf War Illness (manuscript in preparation). In this study, A total of 39 subjects were utilized in these studies; 20 cases identified as having GWI, and 19 healthy controls. Whole blood (2.5 mL) was collected by venipuncture using PAXgene™ Blood RNA collection tubes and total RNA was isolated using the PAXgene Blood RNA Kit. Polyadenilated RNA was purified from total RNA (5 µg) using the Dynabeads® mRNA DIRECT™ Micro Kit and 10 µL of purified mRNA was used to prepare each library. Each respective library was prepared using the Ion total RNA seq kit (Life Technology). Briefly, mRNA was fragmented using RNAse III and purified by nucleic acid binding magnetic beads. The mRNA fragments were then hybridized and ligated with adaptor. The ligation product was reverse transcribed to generate cDNA. Purified cDNA was then amplified, during which time each individual strand was barcoded. At the end of amplification, barcoded cDNA was purified and processed for the Ion P1 Chip on the Ion OneTouch system. Final sequencing was conducted using the Ion Proton NGS system. In total, the output of ten Ion Proton PI chips generated 67 gigabases (Gba) of sequence data between the 39 whole-blood RNA libraries. To maximize high-quality alignments for read alignment, gene identification, and quantification, the raw sequence reads were trimmed to eliminate adapters and low-quality reads and short sequences were removed, yielding 595 million trimmed sequences, with an average sequencing depth of 15 million independent sequence reads per sample and a mean length of 106 nucleotides (nt) per read. 94% of the cleansed reads were successfully aligned to the human genome (GRCh37), using Bowtie2 software [12]. Genomic alignments were then mapped at 25,228 loci with the coding regions of 42,087 NCBI RefSeq transcript models to quantitatively measure global gene expression. 55% of genomic-aligned reads were mapped to the coding sequences of RefSeq gene models. Gene- and isoform-level abundances were calculated using cufflinks [13]. For differential expression testing, the FPKM (Fragments Per Kilobase of exon per Million fragments mapped) quantities were then transformed into pseudo-count approximations by a simple transformation using gene lengths and effective library sizes. Expressed as counts per million (CPM), a baseline filter was applied to select 14,598 genes for which CPM>1 in any 20 of the 39 samples. (A CPM value of 1 corresponded to a count of approximately 10 in this study). This filtering was performed to extract those genes expressed on one of the two samples. The count data of these 14,598 genes were then normalized using upper quartile normalization. Upon normalization, the genes were subjected to a general linear model hypothesis test to determine whether differences between cohorts were statistically significant. Any genes having an unadjusted p-value with $p<0.01$ were used for IPA pathway analysis[14]. IPA pathway analysis then identifies genes that are related through biochemical pathways and calculates the probability that those genes were randomly expressed.

Pathway analysis identified two primary classes of biochemical pathways with differentially expressed genes. These two pathways also have genes that appreciable overlap. The most significantly represented pathway in the PI3K/ART/mTOR signaling pathway (12 of the 123 annotated genes, p value: 1.86×10^{-4}) is given in Table 5.

Table 5. GENE Symbol	Description of genes differentially expressed in the PI3K/ART/mTOR signaling pathway	P value
BCL2	B-cell CLL/lymphoma 2	0.008
BCL2L2	BCL2-like 1	0.010
FOXO1	Forkhead box 01	0.022
INPP5F	Inositol polyphosphate-5-phosphatase	0.011
ITGA4	Integrin, alpha 4 (antigen CD49D)	0.039
JAK1	Janus kinase 1	0.038
JAK3	Janus kinase 3	0.004
NFKBIB	Nuclear factor of kappa light	0.004
PPP2CB	Protein phosphatase 2 catalytic subunit B isozyme	0.008
PPP2R5C	Protein phosphatase 2, regulatory subunit B', gamma	0.047
TP53	Tumor protein p53	0.023
YWHAG	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma	0.028

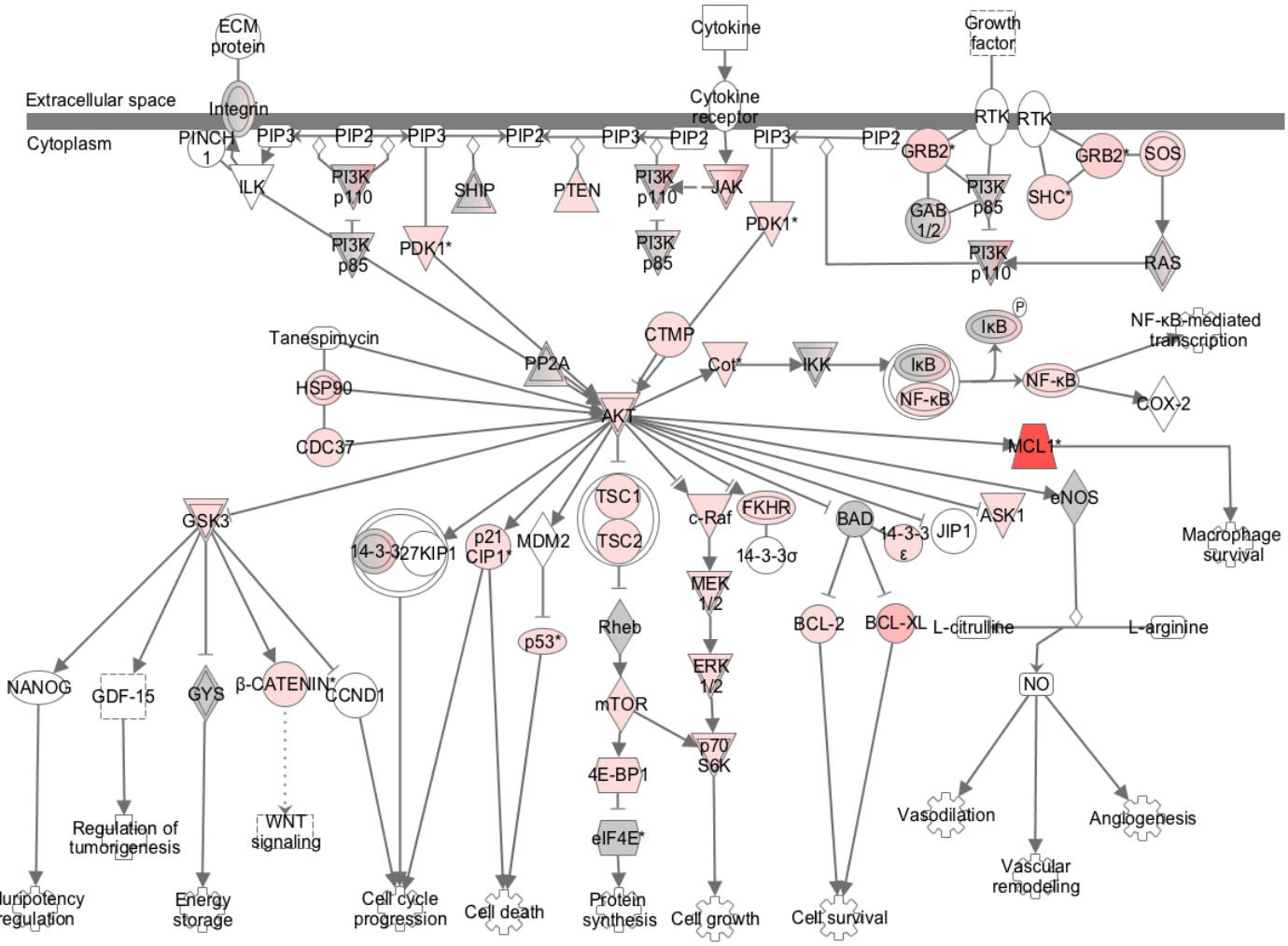

Figure 7. Antigen presentation pathway identified by IPA pathway analysis. Proteins differentially expressed in the PI3K/ART/mTOR signaling pathway as identified by RNAseq. Increasing red color implies a greater level of differential expression.

In addition to the PI3K/ART/mTOR signaling pathway, our RNAseq data also identified overlapping pathways which were significant but to a lesser extent and included: Antigen-presentation pathway (Figure 8); and the Mitrohondial dysfunction pathway and the Production of nitric oxide and reactive oxygen species in macrophages pathway (Figure 9a and b) Select genes of differentially expressed transcripts implicated in these were significant at $p<0.01$.

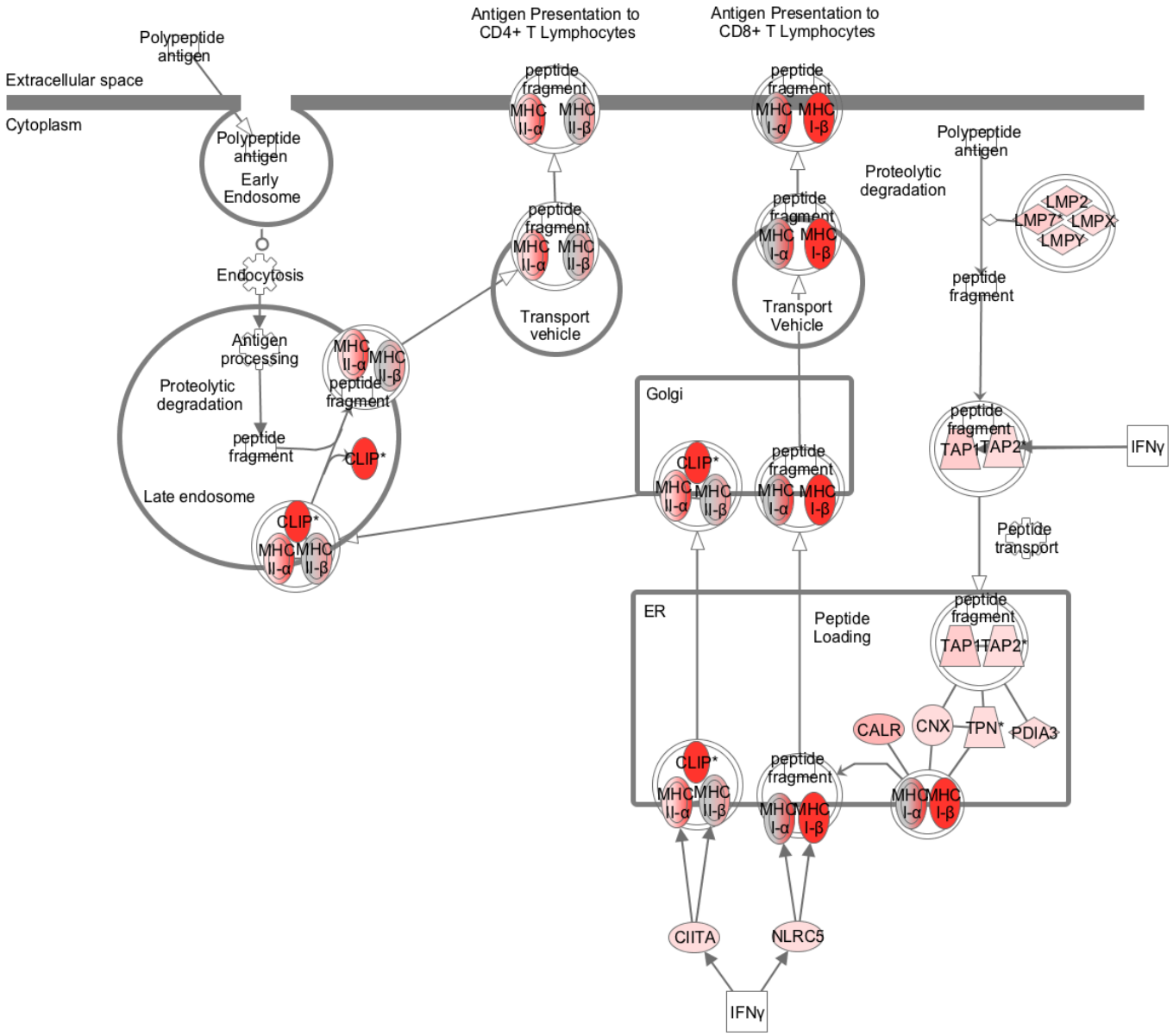

Figure 8. Antigen presentation pathway identified by IPA pathway analysis. Proteins related to the differentially expressed identified by RNAseq are shown as increasing red color given their level of differential expression.

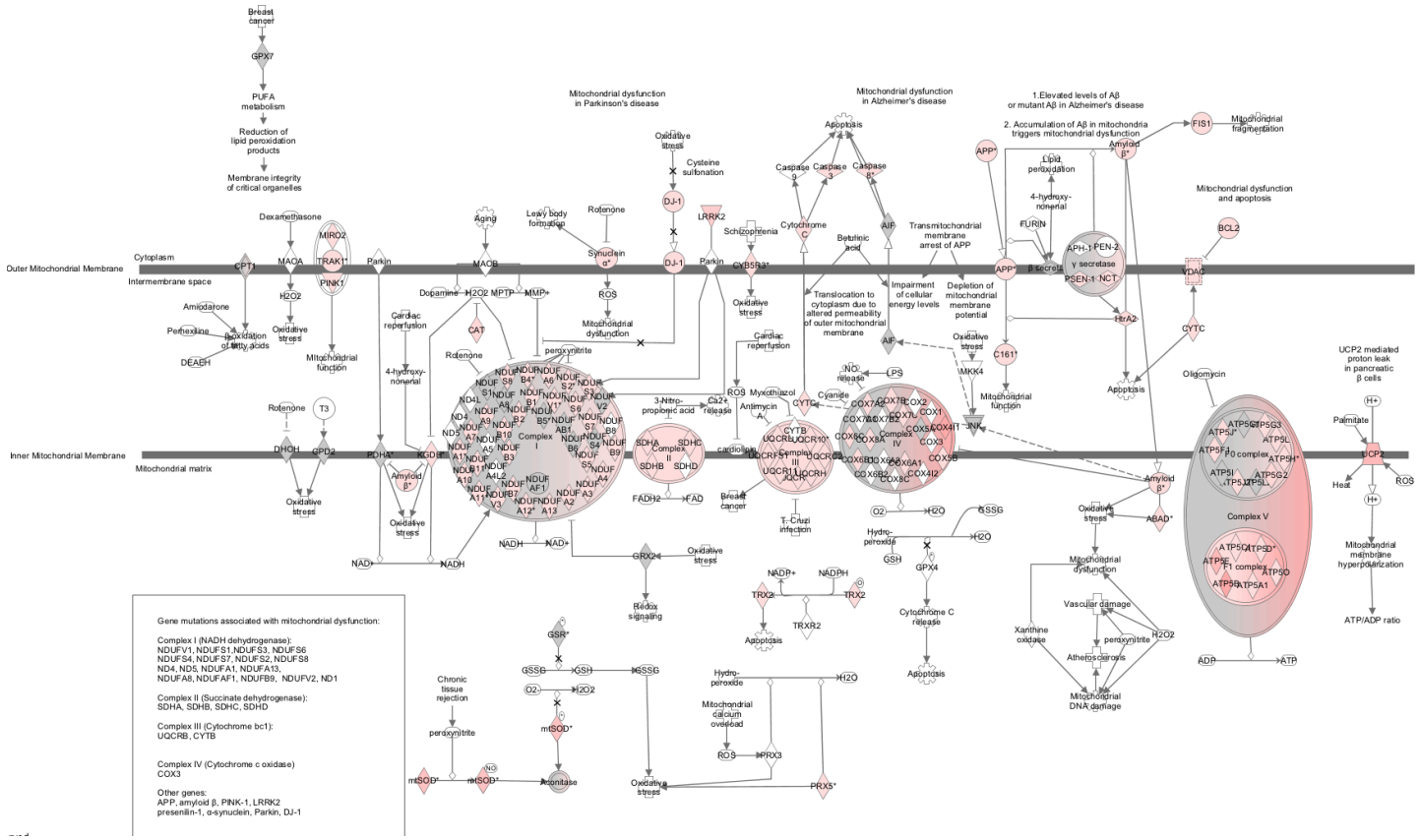

Figure 9a. Mitochondrial dysfunction pathway as identified by IPA pathway analysis. Proteins related to the differentially expressed identified by RNAseq are shown as increasing red color given their level of differential expression.

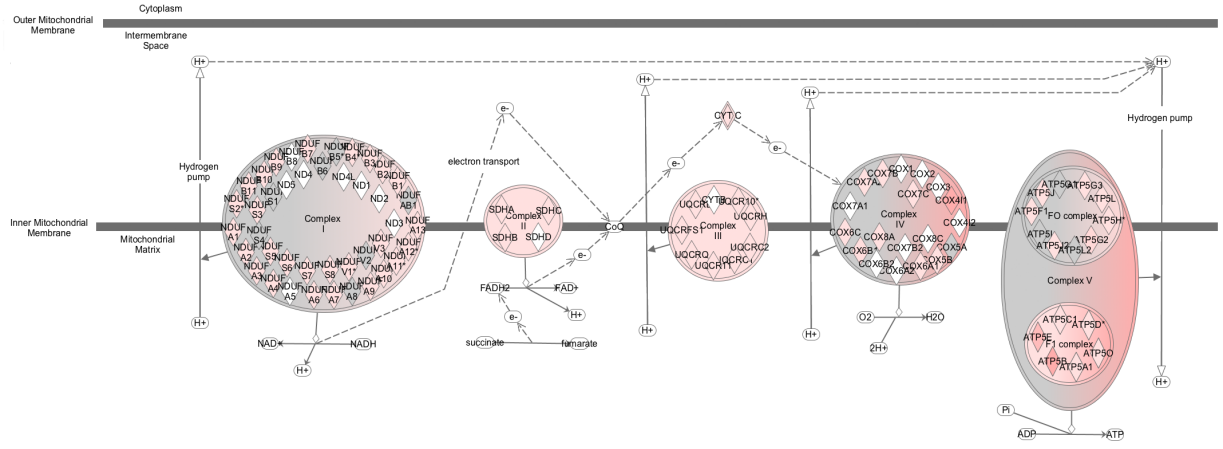

Figure 9b. Oxidative phosphorylation pathway as identified by IPA pathway analysis. Proteins related to the differentially expressed identified by RNAseq are shown as increasing red color given their level of differential expression.

Summary points of this study

1. This study suggests that subjects with GWI are characterized by differential expression of genes common to the PI3K/ART/mTOR signaling pathway as well as to the antigen presenting pathway and the mitochondrial/oxidative phosphorylation pathway.
2. This study further suggests that antigen presenting cells such as plasmacytoid dendritic cells, macrophages and B cells maybe be dysregulated.
3. It should be noted that the results of this study are preliminary and the data analysis will need to be confirmed. We anticipate that a manuscript will be completed prior to the end of the year. This publication was delayed as our bioinformaticist has been on maternity leave for the last two months.

4. KEY RESEARCH ACCOMPLISHMENTS:

As a result of this study:

1. This study suggests that GWI and ME are distinct disease
2. This study supports a Th1/TH17 shift in GWI
3. This study suggests that a ratio of IL-17A and IL-21 can discriminate GWI, ME and healthy controls
4. This study identifies IL-7, IL-4, TNF-α, IL-13, IL-17F, IL-1 as the most important cytokines that contribute to GWI pathology.
5. This study identified 14 cytokines that are differentially expressed in subjects with GWI.
6. This study suggests that immune cells from GWI subjects are hyper-responsive to the lipopolysaccharides produced by Gram-negative bacteria.
7. This study suggests that plasmacytoid dendritic cells are dysregulated in subjects with GWI, potentially at the level of TLR-7.
8. This study suggests that subjects with GWI are characterized by differential expression of genes common to the PI3K/ART/mTOR signaling pathway as well as to the antigen presenting pathway and the mitochondrial/oxidative phosphorylation pathway.
9. This study further suggests that antigen presenting cells such as plasmacytoid dendritic cells, macrophages and B cells maybe be dysregulated.
10. Additionally, our analysis failed to identify a pathogen in subjects with GWI, and further failed to identify mycotoxins in the serum of subjects with GWI suggesting that an exogenous pathogen or environmental fungus is involved with this disease.

5. CONCLUSION:

The goal of this study was to identify pathogens that associate with GWI and additionally to identify biomarkers that may lead to a greater understanding of the pathophysiology of the disease. To fulfill this mandate we took a multi-level approach. We investigated the level of inflammatory cytokines in the serum of GWI subjects and compared these data to subjects with a closely related disease, myalgic encephalomyelitis (ME) also known as chronic fatigue syndrome, and to healthy controls. Previous studies have suggested that GIW and ME are the same disease with different triggers; however, our study suggests this is not the case. However, thought our serum cytokine analysis we were able to identify that GWI is characterized by a TH1/TH17 shift, consistent with the results of Skowera et al. [4]. Additionally, we identified that GWI subjects can be differentiated from ME cases as well as health controls by a ratio of IL-17A/IL-21. Most importantly, this study identified a clear

defect in the ability of immune cells to respond to ssRNA through the activation of TLR-7 and also suggested that the immune system of those with GWI are hyperresponsive to bacterial pathogens.

In order to identify a potential pathogen association with GWI, we screened subjects by next generation sequencing methods (RNAseq) and we additionally screened GWI subjects and controls for the presence of mycotoxins, which are known to inhibits acetylcholinesterase [15]. Both RNAseq for pathogens and mycotoxin screening produced negative results. However, our RNAseq study has potentially provided valuable information that supports the previous studies of Koslik and colleagues [16] who reported that subjects with GWI displayed mitochondrial dysfunction, when compared to matched controls. These data further identify the potential connection to the immune dysfunction as well. It has also been suggested that subjects with GWI have symptoms consistent with autoimmunity [17]. Our data suggests a connection to plasmacytoid dendritic cells, which are heavily implicated in several autoimmune disorders (Reviewed by Lombardi and Khaiboullina [18]).

Although this support for this study has expired, we have elected to extend this study. We have renewed the human subjects protocol and we have resources to continue to expand upon the observations reported here. We anticipate that two manuscripts will be submitted before years, from data already collected but is still being analyzed and using reagents and biological specimens that were made available by this support we anticipate at least two additional manuscripts will be submitted within the next 6 months. We are grateful for the support provided by this award.

6. PUBLICATIONS:

The following peer-reviewed manuscripts have been published or are accepted and acknowledge the support of this award:

Khaiboullina, S.F., DeMeirleir, K.L. Rawat, S., Berk, G.S., Gaynor-Berk, R.S., Mijatovic, T., Blatt, N., Rizvanov, A.A., Young, S.G., **Lombardi, V.C.** Cytokine expression provides clues to the pathophysiology of Gulf War illness and myalgic encephalomyelitis. Cytokine 2014 (accepted)

Lombardi, V.C., Khaiboullina S.F., Rizvanov A.A., Plasmacytoid dendritic cells, a role in neoplastic prevention and progression. Eur J Clin Invest 2014 (accepted) Invited review

Lombardi, V.C., Khaiboullina, S.F. Plasmacytoid dendritic cells of the gut: Relevance to immunity and pathology; Clin Immunol. 2014 Apr 24;153(1):165-177. Review

Properties of Human Lymphocytes Expressing Perforin (PRF1) I.: Natural Killer (NK) Cells Defined as CD3NegPRF1+ Lymphocytes Include CD56Neg Cells in Healthy Subjects". This manuscript was submitted to the Journal Cytometry B and has been accepted with revisions but is still being reviewed, however, I'm not the corresponding author so the revision is not in my control.

Furthermore, have two additional manuscripts that will be submitted before then end of the year and we will forward the manuscripts to the program officer and informed them once the manuscripts have been accepted.

7. INVENTIONS, PATENTS AND LICENSES N/A

8. REPORTABLE OUTCOMES N/A

9. OTHER ACHIEVEMENTS N/A

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18. **Lombardi, V.C. and S.F. Khaiboullina, *Plasmacytoid dendritic cells of the gut: Relevance to immunity and pathology.* Clin Immunol, 2014. 153(1): p. 165-177.**

11. APPENDICIES

Cytokine expression provides clues to the pathophysiology of Gulf War illness and myalgic encephalomyelitis

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Key words: cytokines; Gulf War illness; myalgic encephalomyelitis; cytokines; random forest: interleukin-7

Abstract

Gulf War illness (GWI) is a chronic disease of unknown etiology characterized by persistent symptoms such as cognitive impairment, unexplained fatigue, pervasive pain, headaches, and gastrointestinal abnormalities. Current reports suggest that as many as 200,000 veterans who served in the 1990-1991 Persian Gulf War were afflicted. Several potential triggers of GWI have been proposed including chemical exposure, toxins, vaccines, and unknown infectious agents. However, a definitive cause of GWI has not been identified and a specific biological marker that can consistently delineate the disease has not been defined. Myalgic encephalomyelitis (ME) is a disease with similar and overlapping symptomology, and subjects diagnosed with GWI typically fit the diagnostic criteria for ME. For these reasons, GWI is often considered a subgroup of ME. To explore this possibility and identify immune parameters that may help to understand GWI pathophysiology, we measured 77 serum cytokines in subjects with GWI and compared these data to that of subjects with ME as well as healthy controls. Our analysis identified a group of cytokines that identified ME and GWI cases with sensitivities of 92.5% and 64.9%, respectively. The five most significant cytokines in decreasing order of importance were IL-7, IL-4, TNF-α, IL-13, and IL-17F. When delineating GWI and ME cases from healthy controls, the observed specificity was only 33.3%, suggesting that with respect to cytokine expression, GWI cases resemble control subjects to a greater extent than ME cases across a number of parameters. These results imply that serum cytokines are representative of ME pathology to a greater extent than GWI and further suggest that the two diseases have distinct immune profiles despite their overlapping symptomology.

1. Introduction

Gulf War illness (GWI) and myalgic encephalomyelitis (ME) are complex diseases of unknown etiology. They are often characterized by a constellation of unexplained and overlapping symptoms, which include widespread inflammation, fatigue, multisystemic neuropathology, joint and muscle pain and gastrointestinal pathology [1-3]. Although the two diseases are similar with overlapping symptoms, GWI is a specific term given to returning military veterans and civilian workers of the Persian Gulf War that took place from August 2, 1990 to February 28, 1991. ME is frequently associated with acute flu-like onset as well as noninfectious environmental triggers [4]; whereas, multiple factors including environmental exposure, toxins, vaccines, and unknown infectious agents have been evaluated as potential triggers for GWI [5, 6]. Indeed, GWI and ME have many clinical symptoms in common including long-term and severe fatigue that is not relieved by rest, gastrointestinal disorders, and neurological impairments [2]. Accordingly, it has been suggested that GWI cases meet the diagnostic criteria for ME and, therefore, represent discrete subsets of ME. Currently, there is no pathognomonic marker for either disease as well as no clinical diagnostic test available; for these reasons, diagnosis is mainly based on clinical observation, epidemiological evaluation, and medical anamnesis.

Immunological impairments in subjects with ME have been extensively documented. For example, several researchers have reported abnormalities in natural killer (NK) cell numbers and function [7, 8] as well as abnormalities in serum and plasma cytokine and chemokine levels [9-12]. Natelson et al. observed that levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) in the cerebral spinal fluid of ME cases were lower than in controls and that levels of CXCL8 were elevated in cases with sudden, influenza-like onset when compared to cases with gradual onset or healthy controls [13]. In a study by Zhang and colleagues, two groups of cases who met the case definition for ME were compared to each other; Gulf War veterans who developed their malady after they had returned home from the Gulf and a group of nonveterans who developed the illness sporadically [14]. They reported that Gulf War veterans with ME had a statistically significant increase in total T cells and a lower percentage of NK cells when compared to respective controls. In addition, veterans with ME had higher levels of interleukin (IL)-2, IL-10, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α over that of controls. However, they observed no difference in civilian veterans with ME when compared to controls across a number of immune parameters.

Unquestionably, serum or plasma inflammatory cytokine and chemokine levels are some of the most commonly reported differences between subjects with ME and healthy controls. For example, Maes et al. reported that subjects with ME have significantly higher levels of serum IL-1 and TNF-α when compared to controls [15, 16]. Also, Fletcher and coworkers observed increased levels of serum regulatory and proinflammatory cytokines such as lymphotoxin-α, IL-1α, IL-1β, IL-4, IL-5, IL-6, and IL-12 in ME cases when compared to controls [17]. Other studies have investigated cytokine profiles of subjects with GWI and ME [18]. A Th2 shift is commonly reported for subgroups of ME cases [11, 19], suggesting that those with ME may be hyper-responsive to allergens, toxins, extracellular bacteria, and parasites and hypo-responsive to viruses and intracellular bacteria. Smylie et al. reported a decreased Th2 polarity in females with ME as compared to GWI and suggested that an IL-23/Th17/IL-17 axis could be used to delineate GWI and

ME [18]. Skowera and colleagues reported that, in contrast to asymptomatic Gulf War veterans, symptomatic veterans with "multisymptom illness" displayed an ongoing Th1-type immune activation with significantly elevated levels of IFN-γ and IL-2, in the absence of in vitro stimulation [20]. These studies suggest that although similar in clinical manifestations, GWI and ME potentially present discrete cytokine profiles, which may reflect differences in disease pathogenesis.

Cytokines orchestrate numerous immune functions including activating and prolonging leukocyte proliferation, directing migration, and influencing and shaping leukocyte functional activity. Abnormal leukocyte counts in subjects with GWI and ME may, in fact, be a consequence of dysregulated cytokine control. Subsequently, abnormal leukocyte counts may lead to a disturbed immune response, often manifesting with broad clinical presentations. It may also be suggested that cytokine profiles, which are reflective of the profound immune disturbances in subjects with GWI and ME, might potentially serve as useful biomarkers. A greater understanding regarding cytokine dysregulation in GWI and ME may also help to better understand the pathogenesis of these diseases, thus improving diagnosis, treatment efficacy, and prophylactic measures.

In the present study, we have conducted a comprehensive survey of 77 different cytokines and chemokines in an effort to better understand the immune responses associated with GWI and ME. Our results suggest that Th1 and Th17 cytokines underscore GWI cases, while Th1 and Th2 cytokines as well as a more diverse group of inflammatory cytokines and mononuclear chemoattractant cytokines characterize ME. Additionally, in order to identify the most important cytokines that distinguish these groups and potentially identify underlying pathology, we utilized the machine logic nearest neighbor predictor algorithm Random Forest to analyze these data. The five most significant cytokines identified by our model in decreasing order of importance were IL-7, IL-4, TNF-α, IL-13, and IL-17F. Although our Random Forest analysis produced a cytokine signature that identified ME cases with 92.5% sensitivity, only 64.9% sensitivity was achieved when delineating GWI cases. Furthermore, specificity was only 33.3%, suggesting that with respect to cytokine expression, GWI cases resemble control subjects to a greater extent than ME cases across a number of parameters. These results imply that serum cytokines are representative of ME pathology to a greater extent than GWI and further suggest that the two diseases have distinct immune profiles despite their overlapping symptomology.

2. Materials and methods

2.1 Study subjects

A total of 146 subjects were enrolled in these studies; 67 cases with a confirmed diagnosis of ME, 37 identified as having GWI, and 42 healthy controls. Informed consent was obtained from each participant according to human subjects protocols approved by the University of Nevada Biomedical Institutional Review Board (protocols B12-031 and B12-036). The cases identified as having ME were physician diagnosed and met the Carruthers et al. criteria for ME as well as the 1994 Fukuda et al. criteria [1, 21, 22]. ME subjects were recruited from across the United States and from individuals who sought treatment for ME at the Himmunitas ME/CFS clinic in Brussels Belgium. GWI subjects were recruited by the VA Sierra Nevada Health Care System Medical Center in Reno, Nevada, and were physician diagnosed satisfying the inclusion criteria of having been on

active duty in the military during the Persian Gulf War (Operation Desert Storm: 1990-1991) and symptoms consistent with GWI as defined by the Centers for Disease Control and Prevention (CDC) and Kansas criteria for GWI [23, 24]. Cases were generally representative of the respective populations for each disease based on gender and age.

2.2. Serum samples

Our initial evaluation regarding the method of blood collection indicated that most anticoagulants we tested activated cytokine expression to some level over a 24-h time period (data not shown). The activation was the most pronounced with blood collected on heparin. Given that lymphocytes from ME subjects respond to a greater extent upon stimulation than controls (unpublished observation), this problem would not be normalized even when cases and controls are handled in an identical manner. Additionally, our study required some blood to be shipped overnight; therefore, we chose to conduct our analysis on serum rather than plasma. Whole blood was collected using serum-separator tubes, centrifuged immediately to isolate the serum, and aliquots were made at approximately 24 h post draw and stored at -80 °C until analyzed.

2.3. Cytokine analysis

Serum cytokine levels were analyzed on a Luminex 200 analyzer (Austin, TX) with Bio-Plex (Bio-Rad, Hercules, CA) multiplex magnetic bead-based antibody detection kits according to the manufacturer's instructions. Bio-Plex Pro Human Chemokine panels (40-Plex), Bio-Plex Pro Human Th17 Cytokine panels, Bio-Plex Pro Human Cytokine 27-plex panels, and Bio-Plex Human Cytokine 21-plex panels were used to cover a total of 77 cytokines and chemokines (herein referred to as "cytokines"). For each subject, 50 µl of serum was analyzed and a minimum of 50 beads per cytokine was acquired. Data collected was analyzed using MasterPlex CT control software and MasterPlex QT analysis software (MiraiBio division of Hitachi Software, San Francisco, CA). Standard curves for each cytokine were generated using standards provided by the manufacturer and some samples were analyzed on multiple runs for quality control purposes and to normalize the collective runs.

2.4. Statistical analysis

In order to determine differences in cytokine values and distributions between GWI, ME, and control subjects, we initially performed Kolmogorov-Smirnov tests for normality, which revealed that the data were not normally distributed (data not shown). We therefore used the non-parametric Kruskal–Wallis (K.W.) one-way analysis of variance by ranks to confirm that the three populations did not originate from the same distribution. We then performed a Mann-Whittney (M.W.) analysis to identify differences in medians between GWI and ME cases as well as between GWI cases and controls and ME cases and controls. We additionally conducted Pearson correlation analysis, comparing cytokines to each other within each respective subject population. Finally, we performed classification analysis using the tree-based ensemble machine learning algorithm Random Forest [25]. For this analysis, 500 random trees were built using six predictors for each node, and auto-bootstrap out-of-bag sampling was used for testing the model.

3. Results

3.1. Differential expression of serum cytokines

In these studies, a total of 104 cases (67 ME and 37 GWI) and 42 controls were analyzed for 77 serum cytokines. Subjects classified as having ME were physician diagnosed and fulfilled the criteria described by Carruthers et al. [1, 21] as well as the Fukuda criteria [22]. GWI subjects were physician diagnosed at the VA Sierra Nevada Health Care System Medical Center in Reno, Nevada, and met the inclusion criteria as having been on active duty in the military during the Persian Gulf War (Desert Storm: 1990-1991) and symptoms consistent with GWI as defined by the Centers for Disease Control and Prevention (CDC) and Kansas criteria for GWI. [23, 24]. Subjects' ages ranged from 23 to 81 years (mean age = 58.9 years). For ME cases, the ratio of females to males was approximately 2 to 1 (64% and 36%, respectively); for GWI cases the ratio of males to females was approximately 2 to 1 (64% and 36%, respectively). Controls were of approximately equal proportions (57% male and 43% female).

The K.W. test was initially utilized to compare the individual cytokines concurrently for GWI and ME cases as well as healthy controls. Of the 77 cytokines analyzed, 48 (63%) differed for at least one of the three groups ($p \leq 0.05$), suggesting that the respective cytokine values did not originate from the same distribution (Supplemental data Table 1). We next utilized the M.W. test to compare the two groups of cases with each other and each group of cases with the control group (Supplemental data Table 1). When GWI and ME subjects were compared, 48 cytokines were observed to be significantly different ($p \leq 0.05$). Additionally, when ME cases were compared to healthy controls, 42 cytokines were observed to be differentially expressed, 17 of which were upregulated and 26 were downregulated (Supplemental data Table 2). This is in contrast to only 14 cytokines that differed between GWI cases and controls, 7 of which were upregulated and 7 were downregulated (Table 1.). These observations suggest that, with respect to cytokines, GWI cases resemble healthy controls to a greater extent than they resemble ME cases.

3.2. Th1, Th2, and Th17 cytokine expression

Previous studies have reported that Th1, Th2, and Th17 cytokines, or combinations thereof, characterize GWI and ME. To explore this possibility, we organized these cytokines into three groups (Table 2). For subjects with ME, the Th1 cytokines IFN-γ, IL-2, and IL-12(p75) were upregulated, while the Th2 cytokine IL-5 and IL-9 were downregulated. Paradoxically, the classical Th2 cytokines IL-4, IL-6, IL-10, IL-13 were also upregulated in subjects with ME. For subjects with GWI, the Th1 cytokine IFN-γ was upregulated; however, CXCL8 was slightly downregulated. Additionally, the Th2 cytokines IL-4, IL-13 and IL-25 were all significantly downregulated. For the Th17 cytokines, we observed IL-17F to be significantly downregulated in ME cases, while IL17A and IL17F were both significantly upregulated in GWI cases. Of the 48 cytokines that were differentially expressed in ME, 12 (25%) represented Th1, Th2, or Th17 cytokines. In contrast, of the 14 differentially expressed cytokines observed in GWI subjects (Table 2), 9 (64%) represented Th1, Th2, or Th17 cytokines.

3.3. Cytokine correlation analysis

In addition to T cells, other cells make many of the cytokines typically associated with a Th1, Th2 or Th17 shifts. For instance, the endogenous pyrogen TNF-α is primarily made by activated macrophages, but is also made by most nucleated cells including lymphocytes, fibroblasts and neurons [26, 27]. Likewise, IL-6 is produced by activated macrophages as well as T cells and can act in a proinflammatory or antiinflammatory capacity [28]. Therefore, we conducted correlation analysis, in order to provide additional clues as to which cells produce these cytokines in our study groups. Our results suggest the Th1 cytokines strongly correlate in the ME population but substantially less so for the Th2 cytokines (Table 3.). Additionally, a complete absence of correlation was observed for the Th17 cytokines in the ME group. In contrast to the ME group, only IL-2 and IL-12(p75) showed any significant correlation in GWI. Again, with respect to the GWI group, the Th1 cytokines showed a much weaker correlation and strikingly, TNF-α was negatively correlated with IFN-γ and IL-2, which is in contrast to the ME and control groups (Table 3.). Also, the Th17 cytokines showed a moderately positive correlation in the GWI population. Interestingly, the correlation between IL-17A and IL-21 showed a positive correlation in contrast to that of the control population, which showed a negative correlation.

In addition to the Th1, Th2 and Th17 cytokines, we conducted correlation analysis on the remaining analytes and observed almost perfect correlation ($R^2 \geq 0.90$) between a number of cytokines in the ME group (26 cytokines) but fewer in the GWI group (14 cytokines) (Supplemental data Table 3). Of particular interest, we observed almost perfect correlation between IL-7 / IL-13, IL-7 / FGF, and IL-7 / TNF-α and between IL-3 / IFN-α in the ME group. We also observed near perfect correlation between IL-1α / IL-3, IL-1α / IL-12(p40), IL-1α / IL-2 and IL-1α / IL-22 and between IL-1 / IL-7 and IL-1 / IL-13 in the GWI group.

3.4. Classification of cytokines by importance

Both GWI and ME are diseases with undefined etiology and both are often characterized by aberrant cytokine expression; however, the patterns of cytokine expression appear to be more complex than can be described by a standard Th1, Th2 or Th17 shift. With this in mind, we utilized the machine logic algorithm Random Forest (RF) to analyze our data set and potentially identify the most important cytokines that define these diseases. For this analysis, 500 random decision trees were built using six predictors for each node, and auto bootstrap out-of-bag sampling was employed to test the model. The 20 most significant cytokines for delineation of each group of subjects in order of decreasing importance were IL-7, IL-4, TNF-α, IL-13, IL-17F, IL-1, IL-5, IL-25, CXCL8, VEGF, CCL11, IL12(p75), IL-9, CFS3, IFN-γ, CCL4, IL-6, CCL2, CXCL1, and CXCL10 (Figure 1.). Using only serum cytokines, we were able to achieve sensitivity of 92.5% for delineating ME; however, only 64.9% sensitivity was achieved when delineating GWI with 33.3% overall specificity (Table 3). These data indicate that using serum cytokines alone may not yield an effective diagnostic tool; however, it may provide important clues regarding the underlying pathology of the disease.

4. Discussion

Previous studies of GWI and ME often report that study subjects are characterized by abnormal numerical and functional leukocyte parameters. For example, when compared to healthy

controls, NK cell enumeration and functionality have been reported to be abnormal in both diseases [7, 29]. Additionally, atypical cytokine expression profiles are often reported in association with GWI and ME, although the results are often contradictory. For example, a distinct immune profile of attenuated Th1/Th17 and elevated Th2 responses was reported by Broderick et al., in subjects with ME [9]. However, in another study, Moss et al. observed upregulation of proinflammatory cytokines in the serum of ME cases, suggestive of a Th17 shift [30]. Likewise, cytokine profiling in GWI has been fraught by contradictory results, where predominant Th1 or Th2 immune responses have been reported [14, 20, 31]. Consequently, although differences in serum or plasma cytokines are well documented between cases and controls in both diseases, there is no consensus on a dominant cytokine expression profile for either disease. These conflicting findings may be a result of the heterogeneous nature of these diseases or perhaps a result of different methods of analysis or blood collection procedures. It is also likely that, at any given time, cytokine expression of an individual may change over time, complicating their use as a diagnostic marker.

In this report, we have presented a comprehensive analysis of 77 different cytokines, which to our knowledge represents the largest investigation of serum cytokines in GWI and ME subjects to date. Subjects' blood was collected using serum separator tubes and centrifuged immediately in order to isolate serum cytokines without the use of anticoagulants. By using this method, we have eliminated the possibility that the observed results were subject to leukocyte activation associated with anticoagulants such as heparin or assay interference associated chelating agents like ethylenediaminetetraacetic acid (EDTA).

In this study, we observed differences between cases and controls for 48 of the 77 cytokines investigated, using a confidence interval of 95%. Of the cytokines analyzed, 42 (54.5%) were found to be significantly different between ME cases and healthy controls. In contrast, only 14 cytokines (17.7%) were found to be significantly different between GWI cases and controls. Additionally, when comparing GWI and ME cases, 48 of 77 cytokines were differentially expressed. These data suggest that subjects with GWI and ME are unlikely to represent the same population.

Previous studies have suggested that subjects with GWI and ME may be characterized through the expression of either Th1 or Th2 cytokines. Upon activation, proliferating helper T cells may develop into effector T cells that are often classified as either Th1 or Th2 cells. Th1 immunity is directed against intracellular pathogens such as viruses and mycobacteria, whereas Th2 immunity is typically in response to extracellular pathogens such as fungi and helminths. For these reasons, the cytokines produced by these cells are also referred to as Th1- or Th2-type cytokines. The cytokine expression observed in this study with respect to ME cases, was largely inconsistent with a clear Th1- or Th2-type immune response. For instance, we observed an upregulation of IFN-γ ($p \leq 0.001$) and IL-12(p75) ($p \leq 0.001$) in the absence of an increase in IL-12(p40). These data are consistent with a classic Th1 response [32]. On the other hand, we also observed an upregulation of the IL-10 and IL-4, and when compared to healthy controls ($p \leq 0.0001$), suggestive of a Th2 response. These observations, in conjunction with our correlation analysis, suggest that the Th1 and Th2 cytokines observed in subjects with ME may originate from other immune cells in addition to T cells.

Our data more strongly supports a Th1/Th17 immune polarization in subjects with GWI. Serum cytokine analysis of these subjects showed an upregulation of the Th1 cytokine IFN-γ ($p \leq 0.003$) and the Th17 cytokines IL-17A ($p \leq 0.032$) and IL-17F ($p \leq 0.001$) and a concomitant downregulation of the Th2 cytokines IL-4 ($p \leq 0.014$) and IL-13 ($p \leq 0.001$) when compared to healthy controls. Exposures to such things as toxins, vaccines and unknown infectious agents have been suggested as potential triggers for GWI [5, 6]. Several such triggers have been associated with a Th1/Th17 cytokine shift. For instance, Robbe et al. reported that the occupational exposure to agricultural dust was associated with upregulation of IL-17 and IFN-γ [33], and Harris and coworkers reported that human DCs upregulate IL-17 and IFN-γ in response to the bacteria *B. anthracis* [34]. Additionally, the cationic liposome adjuvant system CAF01, which is commonly used in such vaccines as the trivalent influenza vaccine, is reported to promote a strong and sustained Th1 and Th17 response [35]. Although we cannot say that any of these triggers contribute to GWI, the observed Th1/Th17 shift would be consistent with such triggers.

Little is known regarding the pathophysiology of GWI and ME; nevertheless, the source or class of cytokines produced in subjects with these diseases may provide important clues. Indeed, cytokine profiling has provided valuable knowledge regarding the pathogenesis of other diseases. For example, DeFuria et al. used cytokine profiling to identify the source of inflammatory cytokines associated with type 2 diabetes [36], and Swindle and coworkers utilized cytokine expression data to dissect the psoriatic transcriptome and identified the respective cellular contributions associated with this disease [37]. Additionally, Valeyev and colleagues showed that using a systems model approach; cytokine expression data could be used to provide a quantitative description of immune cell interactions in subjects with psoriasis [38]. In order to identify potential cytokines that may provide information regarding the pathogenesis of GWI and ME, we implemented the machine logic algorithm Random Forest (RF) to analyze our data set. The RF algorithm uses an ensemble of unpruned classification or regression trees produced through bootstrap sampling of the training data set and random feature selection in tree generation. Prediction was made by a majority vote of the predictions of the ensemble. The strength of the analysis was evaluated by an out-of-bag sampling without replacement of the original data. The RF is an attractive method since it handles both discrete and continuous data, it accommodates and compensates for missing data, and it is invariant to monotonic transformations of the input variables. The RF algorithm is uniquely suited for cytokine analysis in that it can handle highly skewed values well and weighs the contribution of each cytokine according to its relatedness with others. Using cytokine expression as input variables and subject status (i.e., GWI case, ME case, and control) as the outcome variable, we identified a group of cytokines that associated with disease status and, therefore, may contribute to the pathogenesis of the disease.

The five most significant cytokines identified by our model in decreasing order of importance were IL-7, IL-4, TNF-α, IL-13, and IL-17F. These cytokines were also identified by significant correlations in our analysis. IL-7 is a hematopoietic growth factor and is important for development, maturation and homeostasis of B, T, and NK cells. Stromal cells of the bone marrow and thymus are the primary source of IL-7; however, is it is also produced to a lesser extent by DCs, hepatocytes, and neurons, but not by lymphocytes [39]. Our data suggest that IL-7 is over-expressed in ME ($p \leq 0.001$) and under-expressed in GWI ($p \leq 0.01$) when compared to healthy

controls. Previous studies suggest that the administration of exogenous of IL-7 in humans leads to the expansion of $CD4^+$ and $CD8^+$ T cells with a concomitant decrease of $CD4^+$ Tregs [40]. Other studies have shown that IL-7-treated animals have reduced numbers of T cells expressing the inhibitory molecules suppressor of cytokine signaling 3 (SOCS3) and programmed cell death protein 1 (PD-1) [41]. Several studies suggest that ME is an inflammatory disease, and multiple reports of individuals with ME expressing autoantibodies [42, 43] and the efficacious treatment of ME cases with the B-cell-depleting drug rituximab [44, 45], suggest that some components of ME pathology may also overlap with those of autoimmunity. Aberrant expression of IL-7 and its receptor has been associated with several autoimmune diseases including inflammatory bowel disease, multiple sclerosis, rheumatoid arthritis, and Sjögren's syndrome (Reviewed in [46]). The upregulation of IL-7 may help explain some of the clinical observations associated with ME such as the inflammatory component or the presentation of autoimmune-like symptoms. In that IL-7 is primarily produced in the bone marrow, but not by lymphocytes, transcriptional studies of bone marrow would be prudent; however, given the difficulty in collecting bone marrow biopsies, deciphering its involvement in these diseases may prove difficult. It is also likely that transcriptional profiling studies that only utilize whole blood will fail to identify an important component to the pathophysiology of these diseases.

Our analysis identified IL-4 and TNF-α as the second and third most important cytokines when delineating ME, GWI, and controls. Both IL-4 and TNF-α were over-expressed in ME ($p \leq 0.0001$) and under-expressed in GWI ($p \leq 0.04$ and $p \leq 0.0001$). IL-4 is a classic Th2 cytokine and promotes the differentiation of naïve helper T cells into Th2 cells. Once differentiated, the Th2 cells can produce additional IL-4 in a positive feedback control loop [47]. The chronic nature of these diseases suggests that Th2 cells are a likely source of serum IL-4; however, our correlation analysis suggests that they may not be the only source. During an inflammatory response, IL-4 production is often accompanied with IL-10 production, which is also upregulated in our ME subjects; however, no statistical difference was observed for IL-10 in GWI subjects. Interestingly, IL-13 was the next most important cytokine in our model. Expression of IL-13 was slightly downregulated in GWI and upregulated in ME. Our analysis showed that IL-13 and TNF-α expression was almost perfectly correlated ($R^2 = 0.918$). IL-13 is an antiinflammatory cytokine and its upregulation may be a response to counter the inflammatory effects of TNF-α. Lastly, our model identified IL-17F as the fifth most important cytokine in differentiating GWI cases, ME cases, and healthy controls. We observed IL-17F to be significantly downregulated in ME cases ($p \leq 0.0001$) and upregulated in GWI cases ($p \leq 0.001$). By increasing the production of inflammatory chemokines, IL-17 is a potent mediator of delayed-type responses and its expression promotes the recruitment of monocytes and neutrophils to the site of inflammation. IL-17F, in particular, is associated with respiratory pathology such as asthma [50].

Our analysis using RF suggests that any combination of the 77 cytokines analyzed in our study may not provide a stand-alone differential diagnosis of GWI and ME. Although the cytokine signature delineated ME cases with 92.5% efficiency, only 64.9% sensitivity was achieved when delineating GWI cases. Furthermore, specificity was 33.3% using cytokines only. Our ongoing research suggests that, by using a combination of cytokines and clinical parameters, we can far exceed the sensitivity and specificity of these results (data not shown). This observation further

suggests that cytokines are useful when stratifying subjects into discrete subgroups. It also suggests that the "catch all" terms of GWI and ME may be overly broad. In light of the heterogeneous nature of these diseases, stratification into subgroups may be mandatory in order to make meaningful progress in understanding the pathophysiology of these diseases.

In conclusion, this study supports an involvement for Th1/Th17 cytokines in GWI and further identifies the cytokines IL-7, IL-4, TNF-α, IL-13, and IL-17F as potentially contributing to the pathogenesis of GWI and ME. This knowledge may provide direction in the development of therapeutic treatments for these diseases.

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Table 1. Cytokines upregulated and downregulated in subjects with Gulf War illness (GWI) when compared to healthy controls

Cytokines upregulated in GWI subjects

Analyte	Group	Minimum	Maximum	Mean	Standard Deviation	P value by M.W.
CCL11	CON	20.7	90.9	47.1	16.4	
	GWI	22.2	252.8	72	48.4	0.008
FGF	CON	3	98.5	20.7	17	
	GWI	9.2	57.7	23.9	11.1	0.026
IFN-γ	CON	2.8	50.4	15.4	10.3	
	GWI	7	47.5	22.4	10.6	0.003
IL-17A	CON	2.5	10.5	7.5	1.5	
	GWI	0.8	277.8	13.6	44.8	0.032
IL-17F	CON	1	143.5	17.9	30.8	
	GWI	9.4	678.8	47.8	113.5	0.001
IL-33	CON	8.2	2850.6	723.3	646	
	GWI	56.5	4379.6	1112.2	883.4	0.011
IL-5	CON	2.4	6.4	5.9	0.9	
	GWI	0.1	24.5	6.4	3.4	0.014

Cytokines downregulated in GWI subjects

Analyte	Group	Minimum	Maximum	Mean	Standard Deviation	P value by M.W.
CCL5	CON	300.4	27934.9	5520.3	4290.5	
	GWI	497.4	10415.4	4059.3	2015	0.031
CXCL8	CON	6.4	3660.2	267.4	620.8	
	GWI	2.2	1413.5	126.3	284	0.032
IL-13	CON	8.1	11.3	9	1.3	
	GWI	8.1	13.9	8.2	1	0.001
IL-25	CON	0	4.7	2.4	2	
	GWI	0	4.7	1.3	1.4	0.04
IL-4	CON	0.1	2	1.1	0.6	
	GWI	0.1	4.6	0.9	0.7	0.041
IL-7	CON	1.4	11.8	5.5	3.2	
	GWI	2.5	18.9	3.7	2.7	0.011
TNF-α	CON	17.6	72.2	23.1	10.2	
	GWI	5.7	20	19.3	3	<0.0001

Table 2. Th1, Th2, and Th17 cytokines expression in subjects with GWI and ME

Analyte	CON	ME	GWI	P value by Mann Whittney ME vs CON	GWI vs CON
Th1 Cytokines					
IFN-γ	15.4	32.4	22.4	0.001	0.003
IL-2	4.7	11.3	3.6	0.062	0.271
TNF-α	23.1	45.4	19.3	<0.0001	<0.0001
IL-12(p75)	20	32.7	21.9	<0.0001	0.246
CXCL8	267.4	191.9	126.3	<0.0001	0.032
IL-18	790.2	726.3	873.2	0.219	0.446
IL-12(p40)	290.1	238.3	309.9	0.089	0.914
Th2 cytokines					
IL-4	1.1	1.7	0.9	<0.0001	0.041
IL-13	9	10.7	8.2	<0.0001	0.001
IL-1β	7.9	14.4	6.6	<0.0001	0.133
IL-25	2.4	5	1.3	<0.0001	0.04
IL-10	10.2	31.7	10.8	0.017	0.223
IL-5	5.9	5.3	6.4	<0.0001	0.014
IL-6	4.3	12.4	3.6	<0.0001	0.202
IL-9	31.7	8	8.7	<0.0001	0.1
Th17 cytokines					
IL-17A	7.5	8.7	13.6	0.053	0.032
IL-17F	17.9	2.9	47.8	<0.0001	0.001
IL-21	30.1	44.5	36.2	0.918	0.081

Table 3. Correlation of cytokines in ME, GWI and controls (values given as R-squared)

Th1 Cytokines ME					Th1 Cytokines GWI					Th1 Cytokines CON				
	IL-12p75	TNFα	IL-2			IL-12p75	TNFα	IL-2			IL-12p75	TNFα	IL-2	
IFNγ	0.944	0.797	0.934		IFNγ	0.408	-0.495	0.424		IFNγ	0.305	0.346	0.339	
IL-2	0.929	0.562			IL-2	0.724	-0.166			IL-2	0.837	0.802		
TNFα	0.758				TNFα	0.012				TNFα	0.534			
Th2 Cytokines ME					**Th2 Cytokines GWI**					**Th2 Cytokines CON**				
	IL-9	IL-6	IL-5	IL-13		IL-9	IL-6	IL-5	IL-13		IL-9	IL-6	IL-5	IL-13
IL-4	0.065	0.282	0.561	0.679	IL-4	0.354	0.152	0.674	0.872	IL-4	-0.011	0.660	-0.742	0.878
IL-13	0.074	0.068	0.946		IL-13	0.249	0.16	0.896		IL-13	-0.121	0.680	-0.633	
IL-5	0.056	0.019			IL-5	0.306	0.117			IL-5	0.113	-0.462		
IL-6	-0.017				IL-6	0.013				IL-6	0.041			
Th17 Cytokines ME					**Th17 Cytokines GWI**					**Th17 Cytokines CON**				
	IL-21	IL-17F				IL-21	IL-17F				IL-21	IL-17F		
IL-17A	-0.016	-0.022			IL-17A	0.349	0.102			IL-17A	-0.544	-0.337		
IL-17F	-0.036				IL-17F	0.592				IL-17F	0.815			

Table 4. Random Forest model statistics

Out-of-Bag Testing Misclassification				
Class	N Cases	N Misclassified	Pct. Error	Cost
ME	67	5	7.46%	0.0746
GWI	37	13	35.14%	0.3514
CON	42	28	66.67%	0.6667

Out-of-Bag Testing Prediction Success					
Actual Class	Total Class	Percent Correct	ME N = 70	GWI N = 46	CON N = 30
ME	67	92.54%	62	1	4
GWI	37	64.86%	1	24	12
CON	42	33.33%	7	21	14
Total:	146				
Average:		63.58%			
Overall% Correct:		68.49%			

FIGURES

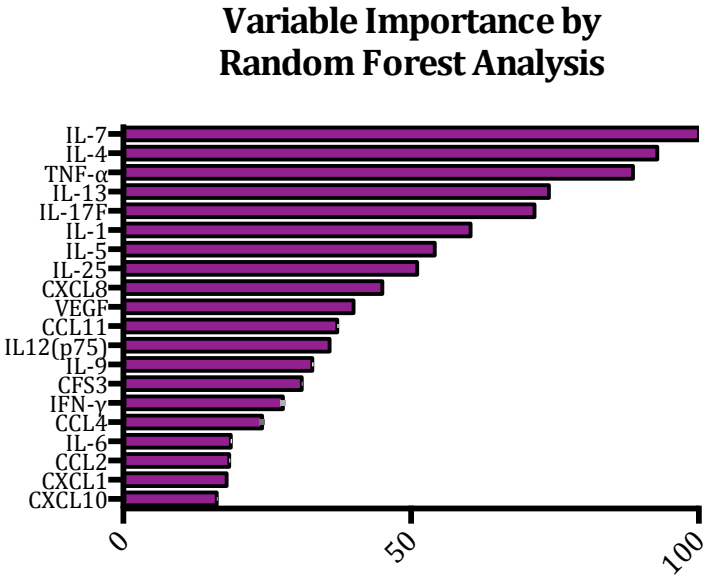

Figure 1. Classification analysis of cytokine data using Random Forest. In order to identify which cytokines most accurately predict disease status of subjects with GWI, ME or controls, Random Forest analysis was implemented whereby 500 random trees were built and six predictors were used at each node. Auto-bootstrap out-of-bag sampling was used for testing the model.

Supplemental Data

Supplemental table 1. Significance of respective cytokines of subjects with Gulf War illness (GWI), myalgic encephalomyelitis (ME), and controls (CON), as determined by the Kruskal–Wallis (K.W.) test and the Mann-Whittney (M.W.) test

Analyte	Group	Minimum	Maximum	Mean	Standard deviation	P value by M.W CON vs. GWI CON vs. ME GWI vs. ME	P value by K.W.
CCL1	CON	39.4	51.9	43.3	2.4	0.32	<0.0001
	ME	40.9	55.1	44.2	2.2	0.012	
	GWI	39.7	47.2	42.8	1.5	<0.0001	
CCL11	CON	20.7	90.9	47.1	16.4	0.008	<0.0001
	ME	16.2	122.7	34.3	20	<0.0001	
	GWI	22.2	252.8	72	48.4	<0.0001	
CCL13	CON	19.2	427.4	135	90	0.227	0.223
	ME	9	344.8	126.9	91.3	0.503	
	GWI	31.8	315.4	144.6	68.7	0.105	
CCL17	CON	33.9	715.4	241.4	143.1	0.067	<0.0001
	ME	36.5	2549.4	233	339.4	0.012	
	GWI	50.5	1313.1	370	287.5	<0.0001	
CCL19	CON	83.4	1082.2	418.5	244	0.246	0.008
	ME	62.2	1738.2	360.3	307.1	0.043	
	GWI	78.5	1190.5	512.5	314.1	0.004	
CCL2	CON	24.6	2022.5	329.7	461.2	0.514	<0.0001
	ME	18.1	10005.2	292.9	1218.9	<0.0001	
	GWI	53.7	2350.1	226.6	370.2	0.004	
CCL20	CON	4.7	32.1	10.8	6.9	0.129	<0.0001

	ME	4.1	241.6	12.9	30.8	0.003	
	GWI	5.6	92.5	13.4	14.4	<0.0001	
CCL21	CON	2050.9	5585.5	3504.6	762	0.076	0.003
	ME	754.3	6547.6	3135.9	1240.2	0.032	
	GWI	1961.6	5668.1	3878.8	1003.2	0.002	
CCL22	CON	602.9	2128.4	1112.8	386.7	0.127	0.009
	ME	263.7	2471.8	999.3	427.5	0.075	
	GWI	329.9	3287.3	1317.4	584.2	0.004	
CCL23	CON	19.1	955.9	375.7	240.4	0.348	0.538
	ME	38.6	1016.4	356.9	198.1	0.674	
	GWI	11.6	739.6	313.1	189.4	0.345	
CCL24	CON	113.9	1435.4	597.8	316.7	0.727	0.958
	ME	75.1	1761.6	640	417.1	0.857	
	GWI	65.7	2998.3	707	635.6	0.86	
CCL25	CON	62.6	787	319.6	160.7	0.523	0.004
	ME	55.3	799.7	254.1	148.1	0.01	
	GWI	93.4	920.1	343	171.8	0.004	
CCL26	CON	7.1	66.2	27.6	12.3	0.488	<0.0001
	ME	1.7	277.4	23.5	33.4	<0.0001	
	GWI	6.3	71.4	29.4	12.3	<0.0001	
CCL27	CON	787.2	2892.5	1411.4	511.5	0.662	0.021
	ME	238.7	2948.3	1235.7	576.6	0.09	
	GWI	566.5	2854.8	1555.1	580.7	0.01	
CCL3	CON	5.7	281.4	43.9	60.9	0.529	0.018
	ME	6.5	1738.8	76.8	241.3	0.011	
	GWI	7.9	789.8	53.6	132.3	0.042	
CCL4	CON	18.9	155.9	68.9	36.7	0.749	<0.0001

	ME	7.4	486.2	52.3	75.3	<0.0001	
	GWI	24.7	223.8	68.3	39.6	<0.0001	
CCL5	CON	300.4	27934.9	5520.3	4290.5	0.031	0.009
	ME	1161.6	25311.4	4491.3	4306.8	0.004	
	GWI	497.4	10415.4	4059.3	2015	0.312	
CCL7	CON	6.6	677	88	115.8	0.976	0.113
	ME	18.9	423.3	60.2	58.1	0.079	
	GWI	8.4	334	75	68.4	0.091	
CCL8	CON	35.1	249.2	95.8	43.3	0.863	0.003
	ME	5.5	214.1	75	39.4	0.007	
	GWI	9.2	240.5	97.1	42.7	0.003	
CSF1	CON	139	1130	426.8	174.6	0.356	0.005
	ME	163	2334	399.6	323.5	0.023	
	GWI	144	1185	481.9	226.1	0.004	
CX3CL1	CON	88.9	408	241.3	84.9	0.879	0.002
	ME	62.9	597.6	203.7	127.1	0.004	
	GWI	83.9	476.3	249.7	93.2	0.002	
CXCL1	CON	93	489.8	232.4	86	0.117	<0.0001
	ME	104.4	1811.3	229.8	249.6	0.009	
	GWI	106.5	572.4	257.4	96.1	<0.0001	
CXCL10	CON	41.3	725.3	197.8	118.8	0.21	<0.0001
	ME	23.5	488.2	143.9	115.3	0.002	
	GWI	71.2	748.4	240.2	140.2	<0.0001	
CXCL11	CON	9.6	432	41.3	64.1	0.163	0.008
	ME	4.3	125	28.3	18.4	0.105	
	GWI	13	247.8	40.4	37.3	0.002	
CXCL12a	CON	48	105	64.4	12.9	0.829	0.481

	ME	44	95	61.7	10.5	0.429	
	GWI	41	415	75.4	60.8	0.245	
CXCL12ab	CON	955.5	4059.2	2367.3	667.5	0.507	0.694
	ME	1079.3	3938.2	2268.7	647.7	0.422	
	GWI	613.4	3925	2283	757.4	0.978	
CXCL13	CON	9.9	53.8	26	10.2	0.316	<0.0001
	ME	4.8	159.5	23.1	22.2	0.002	
	GWI	11.7	439.7	39.8	68.6	<0.0001	
CXCL16	CON	235.7	1105.8	618.1	179	0.91	0.901
	ME	67.3	1408.2	647.8	262.1	0.753	
	GWI	246.6	1255.3	623.1	222.2	0.679	
CXCL2	CON	44.6	911.6	302.1	161.8	0.065	0.18
	ME	47.6	1631.7	374.3	275.7	0.216	
	GWI	80.3	846.6	380.2	193	0.443	
CXCL5	CON	47.7	2238.1	798.1	579.9	0.314	0.244
	ME	38.2	5402.2	722.9	839.9	0.11	
	GWI	47.7	2349.4	678.7	540.9	0.477	
CXCL6	CON	3.1	83.3	48.2	15.3	0.606	0.01
	ME	16.4	225	44.2	26.3	0.018	
	GWI	22.5	93.5	51.6	18.2	0.01	
CXCL8	CON	6.4	3660.2	267.4	620.8	0.032	<0.0001
	ME	4.4	6261.7	191.9	941.4	<0.0001	
	GWI	2.2	1413.5	126.3	284	<0.0001	
CXCL9	CON	66.1	4031.7	355	596	0.083	0.004
	ME	47.3	971.5	257.7	197	0.085	
	GWI	136.6	2815.5	378.1	428.3	0.002	
FGF	CON	3	98.5	20.7	17	0.026	0.088

	ME	4.7	316.3	25.1	38.1	0.415	
	GWI	9.2	57.7	23.9	11.1	0.128	
CFS3	CON	3.8	110.9	26.4	21.4	0.06	<0.0001
	ME	1.6	126.9	16.4	18.8	<0.0001	
	GWI	4.3	76.8	31.6	15.2	<0.0001	
GM-CSF	CON	8.4	106.9	43.4	22.9	0.918	0.518
	ME	3.8	271.5	44.2	43.1	0.3	
	GWI	0.7	92.5	40.2	18.8	0.405	
HGF	CON	217	2639	866.3	532.4	0.871	0.007
	ME	258	1929	656.4	291	0.023	
	GWI	319	2426	836.3	394.1	0.004	
IFN-α	CON	71	479	124.2	62.9	0.613	0.2
	ME	75	232	111.7	30.6	0.242	
	GWI	72	347	124.3	47.5	0.091	
IFN-γ	CON	2.8	50.4	15.4	10.3	0.003	<0.0001
	ME	0.1	911.3	32.4	136	0.001	
	GWI	7	47.5	22.4	10.6	<0.0001	
IL-1	CON	3.7	9.7	4.8	1.1	0.634	<0.0001
	ME	3.8	101.6	7	13.7	<0.0001	
	GWI	4.3	24.3	5.3	3.3	<0.0001	
IL-10	CON	3	84.4	10.2	16.5	0.223	<0.0001
	ME	5.2	932.3	31.7	120.3	0.017	
	GWI	1.2	102.8	10.8	16.5	<0.0001	
IL-12(p40)	CON	93	676	290.1	154.2	0.914	0.136
	ME	90	598	238.3	119.9	0.089	
	GWI	85.5	1854	309.9	289.3	0.112	
IL-12(p75)	CON	9	114.2	20	16.7	0.246	<0.0001

	ME	7.1	549.3	32.7	86.5	<0.0001	
	GWI	13.5	168.2	21.9	25	<0.0001	
IL-13	CON	8.1	11.3	9	1.3	0.001	<0.0001
	ME	8.1	25.8	10.7	2	<0.0001	
	GWI	8.1	13.9	8.2	1	<0.0001	
IL-15	CON	5.7	5.7	5.7	0	0.298	0.087
	ME	5.7	38.4	6.7	5.2	0.17	
	GWI	0.5	5.7	5.6	0.9	0.076	
IL-16	CON	226.6	3602.1	926.2	718	0.724	0.388
	ME	245.8	6130.3	1111.5	1028.8	0.347	
	GWI	166.3	1648.8	738	356.1	0.204	
IL-17A	CON	2.5	10.5	7.5	1.5	0.032	<0.0001
	ME	1.8	60	8.7	6.4	0.053	
	GWI	0.8	277.8	13.6	44.8	<0.0001	
IL-17F	CON	1	143.5	17.9	30.8	0.001	<0.0001
	ME	1	40	2.9	7.2	<0.0001	
	GWI	9.4	678.8	47.8	113.5	<0.0001	
IL-18	CON	209.5	2339	790.2	425.9	0.446	0.126
	ME	221	2971.5	726.3	481.5	0.219	
	GWI	156.5	2281	873.2	475.4	0.053	
IL-1RA	CON	6.7	222.6	50.4	47.3	0.14	0.378
	ME	0.4	10529.9	281.6	1414.7	0.269	
	GWI	7.9	557.4	53.7	106.4	1	
IL-1α	CON	91	602	184.6	104.1	0.073	0.036
	ME	84	511.5	170.8	77.4	0.866	
	GWI	67	1834	250.8	282.4	0.009	
IL-1β	CON	2.6	74.4	7.9	12.1	0.071	0.001

	ME	2.6	640.4	14.4	77.9	<0.0001	
	GWI	2.6	84.6	6.6	13.3	0.133	
IL-2	CON	0.04	31.9	4.7	5.2	0.271	0.01
	ME	0.8	285.2	11.3	36.8	0.062	
	GWI	0.3	15.2	3.6	2.8	0.003	
IL-21	CON	22.1	207	30.1	39	0.081	0.06
	ME	22.1	1465.1	44.5	176.4	0.918	
	GWI	22.1	185.5	36.2	37.6	0.032	
IL-22	CON	23.2	23.2	23.2	0	0.969	0.647
	ME	23.2	23.2	23.2	0	0.213	
	GWI	23.2	134.7	25.5	18.7	0.44	
IL-23	CON	32.3	424.7	95.1	111.6	0.256	0.034
	ME	32.3	1457.5	115.3	237	0.188	
	GWI	32.3	2596.8	174	429	0.009	
IL-25	CON	0	4.7	2.4	2	0.04	<0.0001
	ME	1.1	16	5	2.2	<0.0001	
	GWI	0	4.7	1.3	1.4	<0.0001	
IL-3	CON	77	471	141.8	73.1	0.435	0.207
	ME	70	411	130.7	60	0.435	
	GWI	74.5	804	157.7	125	0.07	
IL-31	CON	1.7	62.6	21.4	14.3	0.185	0.004
	ME	6.6	66.7	31.6	17.2	0.001	
	GWI	1.7	94.1	28	24	0.193	
IL-33	CON	8.2	2850.6	723.3	646	0.011	0.085
	ME	8.2	12366.3	1386.9	2048.7	0.303	
	GWI	56.5	4379.6	1112.2	883.4	0.308	
IL-4	CON	0.1	2	1.1	0.6	0.041	0.207

	ME	0.3	2.9	1.7	0.3	<0.0001	
	GWI	0.1	4.6	0.9	0.7	<0.0001	
IL-5	CON	2.4	6.4	5.9	0.9	0.014	<0.0001
	ME	4.5	27.6	5.3	2.8	<0.0001	
	GWI	0.1	24.5	6.4	3.4	<0.0001	
IL-6	CON	0.04	13.5	4.3	3	0.202	<0.0001
	ME	0.2	235.5	12.4	35.5	<0.0001	
	GWI	0.2	16	3.6	3.6	<0.0001	
IL-7	CON	1.4	11.8	5.5	3.2	0.011	<0.0001
	ME	2.8	45.4	9.8	4.6	<0.0001	
	GWI	2.5	18.9	3.7	2.7	<0.0001	
IL-9	CON	5.2	932.3	31.7	120.3	0.1	<0.0001
	ME	0.1	188.1	8	24.2	<0.0001	
	GWI	2.3	18.4	8.7	3.8	<0.0001	
IL-2RA	CON	88	415	176.7	72.9	0.716	0.667
	ME	72	467	165.8	73.1	0.365	
	GWI	81	379.5	165.8	61.1	0.686	
LIF	CON	121	396.5	217.9	69.9	0.393	0.003
	ME	110.5	460	189	57.4	0.023	
	GWI	106	477	230.5	75.2	0.001	
MIF	CON	667.1	25653.1	6334.1	4391.5	0.533	<0.0001
	ME	386.4	29606.6	4174.9	4994.9	<0.0001	
	GWI	991.2	21912.5	6946.7	4491.2	<0.0001	
PDGF	CON	189.6	2121.7	1095.5	397.4	0.727	0.068
	ME	258.5	2030.7	948.5	423.2	0.039	
	GWI	114.9	2346.4	1059.2	456.3	0.087	
sCD40L	CON	524.8	3903.2	2014.2	812.5	0.113	0.076

	ME	419.9	5094	2203	1017.8	0.484	
	GWI	473.9	4437.7	1755	870.2	0.029	
SCF	CON	119.5	947	459.5	199.1	0.669	0.807
	ME	171	1142.5	451.8	199.6	0.739	
	GWI	173.5	1353	479.3	220	0.555	
SCGF-β	CON	989	11397	4708.1	2303.2	0.546	0.14
	ME	1642	11990	4038.2	2080.1	0.077	
	GWI	1842.5	10637	4327.9	1734.3	0.162	
TNF-α	CON	17.6	72.2	23.1	10.2	<0.0001	<0.0001
	ME	20	1140.9	45.4	142.3	<0.0001	
	GWI	5.7	20	19.3	3	<0.0001	
TNF-β	CON	85.5	495	149	82.4	0.28	0.246
	ME	79	375	137.8	58.3	0.709	
	GWI	79	333.5	155.8	64.7	0.09	
TRAIL	CON	136	652	263.8	94.5	0.644	0.026
	ME	120	408	221.7	59.9	0.011	
	GWI	120.5	555	256.3	87.8	0.072	
VEGF	CON	8.1	183.9	48.1	38.7	0.309	<0.0001
	ME	8.5	81.8	22.1	14.6	<0.0001	
	GWI	8	188.4	54.9	40.2	<0.0001	
β-NGF	CON	68	178	98.5	24.6	0.495	0.101
	ME	66	179.5	92	20.1	0.18	
	GWI	56	359	106.7	48.5	0.042	

Supplemental Table 2. Cytokines differentially expressed in subjects with (ME) when compared to healthy controls.

Cytokines upregulated

Analytes	Group	Minimum	Maximum	Mean	Standard Deviation	P value
CCL1	CON	39.4	51.9	43.3	2.4	
	ME	40.9	55.1	44.2	2.2	0.012
CCL2	CON	24.6	2022.5	226.6	370.2	
	ME	18.1	10005.2	292.9	1218.9	<0.0001
CCL20	CON	4.7	32.1	10.8	6.9	
	ME	4.1	241.6	12.9	30.8	0.003
CCL3	CON	5.7	281.4	43.9	60.9	
	ME	6.5	1738.8	76.8	241.3	0.011
CXCL10	CON	41.3	725.3	197.8	118.8	
	ME	71.2	748.4	240.2	140.2	0.002
IFNγ	CON	2.8	50.4	15.4	10.3	
	ME	0.1	911.3	32.4	136	0.001
IL-1	CON	3.7	9.7	4.8	1.1	
	ME	3.8	101.6	7	13.7	<0.0001
IL-10	CON	3	84.4	10.2	16.5	
	ME	5.2	932.3	31.7	120.3	<0.017
IL-13	CON	8.1	11.3	9	1.3	
	ME	8.1	25.8	10.7	2	<0.0001
IL-1β	CON	2.6	74.4	7.9	12.1	
	ME	2.6	640.4	14.4	77.9	<0.0001
IL-25	CON	0	4.7	2.4	2	
	ME	1.1	16	5	2.2	<0.0001
IL-31	CON	1.7	62.6	21.4	14.3	
	ME	6.6	66.7	31.6	17.2	0.001
IL-4	CON	0.1	2	1.1	0.6	
	ME	0.3	2.9	1.7	0.3	<0.0001
IL-6	CON	0.04	13.5	4.3	3	
	ME	0.2	235.5	12.4	35.5	<0.0001
IL-7	CON	1.4	11.8	5.5	3.2	
	ME	2.8	45.4	9.8	4.6	<0.0001
IL12(p75)	CON	9	114.2	20	16.7	
	ME	7.1	549.3	32.7	86.5	<0.0001
TNF-α	CON	17.6	72.2	23.1	10.2	
	ME	20	1140.9	45.4	142.3	<0.0001

Cytokines downregulated

Analyte	Group	Minimum	Maximum	Mean	Standard Deviation	P value By M.W.
CCL11	CON	20.7	90.9	47.1	16.4	

	ME	16.2	122.7	34.3	20	<0.0001
CCL17	CON	33.9	715.4	241.4	143.1	
	ME	36.5	2549.4	233	339.4	0.012
CCL19	CON	83.4	1082.2	418.5	244	
	ME	62.2	1738.2	360.3	307.1	0.043
CCL21	CON	2050.9	5585.5	3504.6	762	
	ME	754.3	6547.6	3135.9	1240.2	0.032
CCL25	CON	62.6	787	319.6	160.7	
	ME	55.3	799.7	254.1	148.1	0.01
CCL26	CON	7.1	66.2	27.6	12.3	
	ME	1.7	277.4	23.5	33.4	<0.0001
CCL3	CON	5.7	281.4	43.9	60.9	
	ME	6.5	1738.8	76.8	241.3	0.011
CCL4	CON	18.9	155.9	68.9	36.7	
	ME	7.4	486.2	52.3	75.3	<0.0001
CCL5	CON	300.4	27934.9	5520.3	4290.5	
	ME	1161.6	25311.4	4491.3	4306.8	0.004
CCL8	CON	35.1	249.2	95.8	43.3	
	ME	5.5	214.1	75	39.4	0.007
CSF1	CON	139	1130	426.8	174.6	
	ME	163	2334	399.6	323.5	0.023
CSF3	CON	3.8	110.9	26.4	21.4	
	ME	1.6	126.9	16.4	18.8	<0.0001
CX3CL1	CON	88.9	408	241.3	84.9	
	ME	62.9	597.6	203.7	127.1	0.004
CXCL1	CON	93	489.8	232.4	86	
	ME	104.4	1811.3	229.8	249.6	0.009
CXCL13	CON	9.9	53.8	26	10.2	
	ME	4.8	159.5	23.1	22.2	0.002
CXCL6	CON	3.1	83.3	48.2	15.3	
	ME	16.4	225	44.2	26.3	0.018
CXCL8	CON	6.4	3660.2	267.4	620.8	
	ME	4.4	6261.7	191.9	941.4	<0.0001
HGF	CON	217	2639	866.3	532.4	
	ME	258	1929	656.4	291	0.023
IL-17F	CON	1	143.5	17.9	30.8	
	ME	1	40	2.9	7.2	<0.0001
IL-5	CON	2.4	6.4	5.9	0.9	
	ME	4.5	27.6	5.3	2.8	<0.0001
IL-9	CON	5.2	932.3	31.7	120.3	
	ME	0.1	188.1	8	24.2	<0.0001
LIF	CON	121	396.5	217.9	69.9	
	ME	110.5	460	189	57.4	0.023

MIF	CON	667.1	25653.1	6334.1	4391.5	
	ME	386.4	29606.6	4174.9	4994.9	<0.0001
PDGF	CON	189.6	2121.7	1095.5	397.4	
	ME	258.5	2030.7	948.5	423.2	0.039
TRAIL	CON	136	652	263.8	94.5	
	ME	120	408	221.7	59.9	0.011
VEGF	CON	8.1	183.9	48.1	38.7	
	ME	8.5	81.8	22.1	14.6	<0.0001

Supplemental Table 3. Cytokines by Pearson correlation in subjects with ME, GWI and healthy controls shown for cytokines where at least one member is $R^2 \geq 0.90$

Analytes	Group	R^2	Analytes	Group	R^2
IL-1/IL-6	**ME**	**0.973**	IL-5/TNF-α	**ME**	**0.956**
	GWI	0.215		GWI	-0.466
	CON	0.354		CON	0.097
IL-1/IL-7	ME	0.284	IL-7/IL-13	**ME**	**0.992**
	GWI	**0.961**		**GWI**	**0.967**
	CON	0.151		**CON**	**0.971**
IL-1/IL-13	ME	0.304	IL-7/FGF	**ME**	**0.916**
	GWI	0.987		GWI	0.024
	CON	0.146		CON	-0.323
IL-1/ IL-1β	**ME**	**0.856**	IL-7/TNF-A	**ME**	**0.934**
	GWI	0.102		GWI	-0.643
	CON	**0.915**		CON	-0.005
IL-1α /IL-3	ME	0.470	CXCL8/Gro-a	**ME**	**0.933**
	GWI	**0.902**		GWI	0.534
	CON	0.128		CON	0.644
IL-1α /IL-12(p40)	ME	0.573	CXCL8/MIP-3α	**ME**	**0.966**
	GWI	**0.932**		GWI	0.097
	CON	0.277		CON	0.493
IL-1α /TNF-β	**ME**	**0.845**	IL-9/IL-10	ME	0.014
	GWI	0.696		GWI	0.187
	CON	**0.933**		**CON**	**0.969**
IL-1β /MIP-1α	ME	0.871	IL-10/IL-12(p75)	**ME**	**0.928**
	GWI	**0.958**		**GWI**	**0.952**
	CON	0.888		**CON**	**0.912**
IL-1β /SDF-1A	ME	-0.060	IL-10/IFNγ	**ME**	**0.947**
	GWI	**0.927**		GWI	0.417
	CON	0.250		CON	0.216
IL-1RA/IL-2	ME	0.973	IL-12p75/IFNγ	**ME**	**0.944**

	GWI	0.827		GWI	0.408
	CON	0.107		CON	0.305
IL-1RA/IL-10	**ME**	**0.983**	IL-13/FGF	**ME**	**0.918**
	GWI	0.765		GWI	0.019
	CON	0.072		CON	-0.358
IL-1RA/IL-12P75	**ME**	**0.925**	IL-13/TNF-α	**ME**	**0.947**
	GWI	0.839		GWI	-0.645
	CON	0.182		CON	-0.039
IL-1β /MIP-3α	**ME**	**0.949**	NGF/IFNα	ME	0.708
	GWI	0.217		**GWI**	**0.957**
	CON	0.628		CON	0.390
IL-1RA/IFNγ	**ME**	**0.981**	NGF/SDF-1a	ME	0.802
	GWI	0.427		**GWI**	**0.957**
	CON	0.113		CON	0.863
IL-2/IL-10	**ME**	**0.997**	Eotaxin-3/MCP1	**ME**	**0.951**
	GWI	0.638		GWI	0.001
	CON	0.807		CON	0.341
IL-2/IL-12P75	**ME**	**0.929**	Eotaxin-3/TARC	**ME**	**0.928**
	GWI	0.724		GWI	0.195
	CON	0.837		CON	0.379
IL-2/IFNγ	**ME**	**0.934**	Gro-a/MIP-1β	**ME**	**0.900**
	GWI	0.424		GWI	0.258
	CON	0.339		CON	0.302
IL-3/IFN-α	**ME**	**0.932**	IFN-a/SDF-1a	ME	0.618
	GWI	0.696		**GWI**	**0.900**
	CON	0.936		CON	-0.242
IL-3/IL-12P40	ME	0.764	I-TAC/MIG	ME	0.469
	GWI	**0.968**		**GWI**	**0.995**
	CON	0.852		**CON**	**0.964**
IL-4/IL-7	ME	0.677	MIP-1α /MIP-3α	**ME**	**0.900**
	GWI	**0.903**		GWI	0.191
	CON	**0.916**		CON	0.546
IL-5/IL-7	**ME**	**0.943**	MIP-3β /TARC	ME	0.368
	GWI	0.794		**GWI**	**0.904**
	CON	-0.719		**CON**	**0.943**
IL-5/IL-13	**ME**	**0.946**	IL-5-FGF	**ME**	**0.938**
	GWI	**0.900**		GWI	0.037
	CON	-0.633		CON	0.973

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REVIEW

Plasmacytoid dendritic cells of the gut: Relevance to immunity and pathology

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Abstract Plasmacytoid dendritic cells (pDCs) are bone marrow-derived immune cells with the ability to express copious amounts of type I and III interferon (IFN) and can differentiate into antigen-presenting dendritic cells as a result of stimulation by pathogen-derived nucleic acid. These powerful combined functionalities allow pDCs to bridge the innate and adaptive immune systems resulting in a concerted pathogen response. The contribution of pDCs to gastrointestinal immunity is only now being elucidated and is proving to be a critical component in systemic immunity. This review will explore the immunology of pDCs and will discuss their involvement in human disease and tolerance with an emphasis on those in the gastrointestinal lymphoid tissue.

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1. Introduction

1.1. Note from the authors

Since the identification of pDCs as a discrete class of immune cells, significant progress has been made in understanding their developmental process and the mechanisms by which they respond to pathogens. Although we will briefly discuss these subjects, the primary purpose of this review is to emphasize the role of pDCs in gastrointestinal immunity and gut-related pathology. Therefore, we would refer the reader to a number of excellent reviews such as Reizis et al. [1], Fitzgerald-Bocarsly et al. [2], or Lande and Gilliet [3], which provide a comprehensive summary of the development and mechanisms of pDC functionality.

1.2. Identification of pDCs as unique populations of immune cells

Dendritic cells (DCs) are antigen-presenting cells that sense pathogens and present pathogen-derived peptides to T and B cells, thus triggering and influencing adaptive immune responses. In humans, dendritic cells are most commonly divided into two classes: plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (cDCs) [4,5]. cDCs can be further subdivided into five populations based upon their expression of the surface markers CD1c, CD16, or BDCA-3 [6]. Each subtype of cDC has been reported to display significant transcriptional differences, likely reflective of their differences in antigen-uptake, signaling, and migration [7]. pDCs are a unique population of bone-marrow-derived immune cells that upon activation by pathogen-derived nucleic acid produce large amounts of type I and type III IFN as well as proinflammatory cytokines [8,9]. Accordingly, pDCs play a pivotal role in bridging the innate and adaptive immune systems. Although the first unequivocal characterization of pDCs was relatively recent, Lennert and Remmele first described pDCs in 1958 as a subset of cells with plasma cell-like morphology observed in lymph nodes (LNs) [10]. In consideration of their morphology, and their expression of the T cell marker CD4 [11] and monocyte markers such as CD123 and CD68 [10,12], these cells came to be known as plasmacytoid T cells or plasmacytoid monocytes. Twenty years after Lennert and Remmele first described the plasmacytoid T cells, Trinchieri and colleagues identified a subset of non-T cell lymphocytes by their antiviral activity and their ability to activate natural killer (NK)-cell-mediated cytotoxicity through the production of IFN-α [13]. Those cells were subsequently referred to as natural IFN-producing cells. Ultimately, independent research conducted in the laboratories of Liu [14] and Colonna [8] confirmed that the plasmacytoid T cells identified by Lennert and Remmele and the natural IFN-producing cells identified by Trinchieri and colleagues were one and the same.

2. Development, distribution and morphology

2.1. Classic dendritic cells vs. plasmacytoid dendritic cells

pDCs share many key features with cDCs, to which they are related; therefore, it is useful to use cDCs as a point of reference when discussing pDCs. Both pDCs and cDCs originate from a common hematopoietic progenitor and differentiate through a pathway that involves FMS-related tyrosine kinase 3 (FLT3L)-induced signaling [15,16]. Although the two cell populations may originate from a common bone marrow precursor, pDCs diverge down an alternative developmental path in a process that likely requires the constitutive expression of the pDC-specific transcription factor E2-2 as well as the Runt family transcription factor Runx2 [17–20]. The migration and distribution patterns also differ between the two classes of dendritic cells. cDC precursors travel via the bloodstream to the lymphoid organs and peripheral tissue where they develop into immature resident and migratory DCs, respectively [4]. These immature cDCs are committed to antigen sampling and are characterized by low-level expression of T cell costimulatory molecules and major histocompatibility complex (MHC) class II [5]. They may remain in the resident tissue until they encounter an activation signal, typically as a result of the engagement of Toll-like receptors (TLRs) [21]. TLRs are transmembrane receptors that recognize repeating molecular motifs conserved within a specific class of microbe, such as the lipopolysaccharide of Gram-negative bacteria or the unmethylated CpG DNA common to many microbial genomes [22,23]. Upon microbial activation, these immature cDCs will migrate to the LNs where they undergo significant changes that result in the development of their

mature phenotype [21]. In the absence of pathogen stimuli, cDCs can migrate to the LNs under steady state conditions [24], a property that is necessary to fulfill their mandate in the presentation of self-antigens while establishing immune tolerance [25].

2.2. Development, migratory properties and morphology of pDCs

In contrast to the development and migratory properties of cDCs, pDCs fully develop in the bone marrow and subsequently travel via the bloodstream to the thymus and all secondary lymphoid organs through high endothelial venules under steady-state conditions [26,27] in a process that is also dependent on Runx2 as well as the CCR5 (CD195) receptor [19]. Prior to stimulation by pathogen-derived molecules, fully developed pDCs are often referred to as "pre-pDCs"[4] or "resting" pDCs [28]. In the steady state, pDCs can migrate to specific tissue such as the LNs or gut, under the control of chemokine receptors including CCR7 (CD197) and CCR9 (CD199) and their corresponding ligands CCL19 (MIP-3β), CCL21 (6Ckine), and CCL25 (TECK), respectively (Table 1) [29–32]. This migration is essential for presentation of self-antigens and thus for promotion of immune tolerance [23,33,34]. Whereas cDCs migrate from sites of infection to the LNs and subsequently activate naïve T cells, few pDCs appear to do so and only in the late stages of infection [35].

pDCs are unique among immune cells in that they occupy two discrete "professional" roles, one as effector cells in consideration of their consummate production of type I and type III IFN and the other as antigen-presenting cells (APCs) [28]. These two specialized roles exist at distinct functional stages that are characterized by fundamentally different morphologies. Resting pDCs have a morphology resembling antibody producing plasma cells, hence the term "plasmacytoid". Once activated, pDCs undergo profound morphological and functional changes to transform from IFN-producing cells into classical antigen-presenting dendritic cells, capable of stimulating naïve T cells and possessing classic

Table 1 Receptors and ligands of human plasmacytoid dendritic cells.

Receptor	Alt. name	Ligand	Location	Function in pDCs
CD4	CD4	MHC II	Cell membrane	Co-receptor, upregulated upon CpC stimulation
IL3RA	CD123	Interleukin 3 (IL-3)	Cell membrane	Promotes differentiation into cDCs
BDCA-2 [a]	CD303	C-type lectin	Cell membrane	C-type lectin receptor, IFN production blocked by mAbs
ILT7 [a]	CD85g	BST2	Cell membrane	Negatively regulates pDC function, IFN production blocked by mAb
LAMP4	CD68	Lectins or selectins	Endosomal memb.	Tissue homing, promotes phagocytosis
B7-1	CD80	CD28 and CTLA-4	Cell membrane	Co-stimulatory
B7-2	CD86	CD28 and CTLA-4	Cell membrane	Co-stimulatory
TNFRSF5	CD40	CD154 (CD40L)	Cell membrane	Co-stimulatory, activation, TD class switch recombination
FLT3	CD135	FLT3L	Cell membrane	Regulates hematopoiesis
Toll-like receptor 7	TLR-7	Single stranded RNA	Endosomal memb.	Induction of type I interferons and proinflammatory cytokines
Toll-like receptor 9	TLR-9	CpG DNA	Endosomal memb.	Induction of type I interferons and proinflammatory cytokines
RIG-I	DDX58	dsRNA	Cytosolic	Induction of type I interferons and proinflammatory cytokines
MDA-5	IFIH1	dsRNA	Cytosolic	Induction of type I interferons and proinflammatory cytokines
CCR5	CD195	CCL5, CCL3, CCL4	Cell membrane	Gut homing
CCR7	CD197	CCL19, CCL21	Cell membrane	LN homing
CCR9	CD199	CCL25	Cell membrane	Expressed on IE and LP
PKR	EIF2AK2	dsRNA	Cytosolic	Innate immune response to viral infection
LY75	CD205	C-type lectin	Cell membrane	Putative antigen uptake receptor
αEβ7 integrin	CD103	E-cadherin	Cell membrane	Gut homing (upregulated in HIV infection)
β2 integrin	CD18	ICAM-1, FHL2, PSCD1	Cell membrane	Gut homing, pairs with CD11a, b, c, and d
α4β7 integrin	LPAM-1	MadCAM-1	Cell membrane	Gut homing
P-selectin	CD62P	P-selectin glycoprotein lig-1	Cell membrane	Gut homing
CXCR4	CD184	CXCL12	Cell membrane	Chemotatic receptor, co-receptor for HIV
TNFRSF13B	CD267	TNFSF13, BAFF	Membrane (B-cells)	Ligands produced by pDCs, T-cell independent IgA induction

[a] Specific to pDCs.

dendritic morphology. Human peripheral blood pDCs uniquely express the blood dendritic cell antigen-2 (BDCA-2) (also called CD303) and the immunoglobulin-like transcript 7 (ILT7) [36,37]. Additionally, they express CD4 and CD123, but lack expression of CD14 [36]. In contrast, murine pDCs can be defined as $CD11c^{int}$ CD45R/$B220^{+}$ BST2/mPDCA-1^{+}[27]. Recently, a subclass of human pDCs was reported to express the neural cell adhesion molecule CD56, a common NK-cell marker, in response to activation with adult tick-borne encephalitis vaccine FSME [38]. The expression of CD56 was reported to coincide with the presence of other cytotoxic molecules, such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and granzyme B. This observation suggests an additional role for pDCs as cytolytic effector cells, independent of their traditional rolls in type I IFN-production and antigen presentation.

3. Activation and cytokine production through Toll-like receptor engagement

3.1. Type I IFN and inflammatory cytokines

pDCs are the primary producers of type I IFN, having the capacity to produce all isoforms, including IFN-β, IFN-ε, IFN-κ, IFN-ω, and all 12 subtypes of IFN-α. Additionally, they have the ability to produce a significant amount of the type III IFNs: IFN-λ1 and -λ2 (also known as interleukin (IL)-29 and IL-28A, respectively); however, they produce little if any IFN-λ3 (IL-28B) [39–43]. Although accounting for only 0.1 to 0.5% of peripheral blood cells, pDCs are responsible for over 95% of type I IFN produced by circulating lymphocytes [14], firmly establishing their key role as professional IFN-producing cells. In addition to IFN, pDCs also have the ability to produce a number of inflammatory cytokines and chemokines including IL-6, TNF-α, CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CXCL8 (IL-8), and CXCL10 (IP-10) [44–46]. The production of IFN, as well as inflammatory cytokines and chemokines, is a result of a signaling cascade, which initiates through the engagement of endosomally located TLR-7 and TLR-9, by single stranded RNA [47,48] or non-methylated DNA and CpG oligodeoxynucleotides (CpG-DNA) [49,50], respectively (Fig. 1). Similar to all other TLRs (with the notable exception of TLR-3), TLR-7 and TLR-9 employ the universal adapter protein MyD88 (myeloid differentiation primary response 88), which acts via NF-κB and IRF7, to initiate transcription of inflammatory cytokines and IFN, respectively (see Bao and Lius [51] for a comprehensive review of TLR signaling and control in pDCs).

In addition to activation of TLR-7 and TLR-9 by ssRNA and CpG DNA, respectively, pDCs are also reported to produce type I IFN in response to dsRNA [45]. The production of IFN in response to dsRNA, however, is unlikely to be related to TLR engagement in that pDCs are reported to express little if any dsRNA-sensing TLR-3 [52,53]. Previous studies attribute the ability of cDCs to produce type I IFN in response to dsRNA through the engagement of TLR-3 as well as through the cytosolic retinoic acid-inducible gene (RIG)-like helicases RIG-I and melanoma differentiation antigen 5 (MDA5) [54,55]. Kramer et al. reported that human pDCs express RIG-I and MDA5 transcripts as well as protein to a greater extent than that of cDCs, suggesting the possibility of their involvement in dsRNA-triggered IFN production [71]. However, using murine RIG-I knockout model, Kato and coworkers reported that RIG-I and MDA5 did not contribute to the ability of pDCs to respond to dsRNA [55]. Additionally, Ruscanu et al. reported that bluetongue virus, a dsRNA virus, activated type I IFN in purified ovine pDCs in a TLR-7/8-independent and MyD88-dependent manner. Furthermore, using inhibitors of protein kinase R (PKR), a soluble dsRNA sensor, they showed that IFN production could be ablated, supporting a PKR involvement in the dsRNA response by pDCs. These discrepancies may be species related or perhaps they suggest that RIG-I and MDA5 may play different roles in pDCs and cDCs.

3.2. Spatiotemporal engagement of Toll-like receptor 7 and 9

Although TLR-7 and TLR-9 engagement, by their respective ligands, leads to the expression of type I IFN in a MyD88-dependent manner, their response, by which they produce IFN, is not necessarily equivalent. For instance, Hilyer and coworkers showed that in pDCs, TLR-9 engagement by CpG DNA results in the expression of all forms of IFN; whereas, treatment of pDCs with the TLR-7 agonist Imiquimod leads to activation of all forms of type I IFN except IFN-α7 [40]. Although stimulation of TLR-7 or TLR-9 leads to IFN and inflammatory cytokine production, the spatiotemporal engagement of these receptors determines the product of their activation [56,57]. Type A CpGs, which activate TLR-9 and are retained in the early endosomes, together with the MyD88-IRF-7 complex, induce high IFN-α production from pDCs, but type A CpGs are weak activators of TLR-9-dependent NF-κB signaling. In contrast, type B CpGs, which are preferentially retained in late endosomes, are weak activators of IFN but trigger a robust inflammatory cytokine response. The ability of pDCs to retain TLR-activating ligands is a principal factor that contributes to their unique ability to mount a rapid and massive IFN response. Additionally, while IRF-7 is expressed at low levels in most cells and is only upregulated upon microbial infections, high-level expression of IRF-7 is constitutive and unique to pDCs and contributes significantly to their ability to mount a rapid IFN response [58–60].

4. Antigen presentation

4.1. Presentation of exogenous and endogenous antigens

pDCs are widely accepted as "professional" antigen presenting cells and their abilities in this regard have been comprehensively reviewed by Villadangos and Young [61]; therefore, we will briefly recapitulate some of their conclusions and address current developments regarding this subject. As "professional" APCs, cDCs have the capacity to capture, process, and present antigens in order to activate naïve T cells, a process typically referred to as "priming". Exogenous antigens are internalized specifically by phagocytosis and receptor-mediated endocytosis or nonspecifically along with extracellular fluid in the process of macropinocytosis [5]. Exogenous antigens can also be internalized upon infection of the APC by a pathogen such as a virus. Once taken up by the cDC, antigens are partially digested and loaded onto MHC molecules for presentation. The

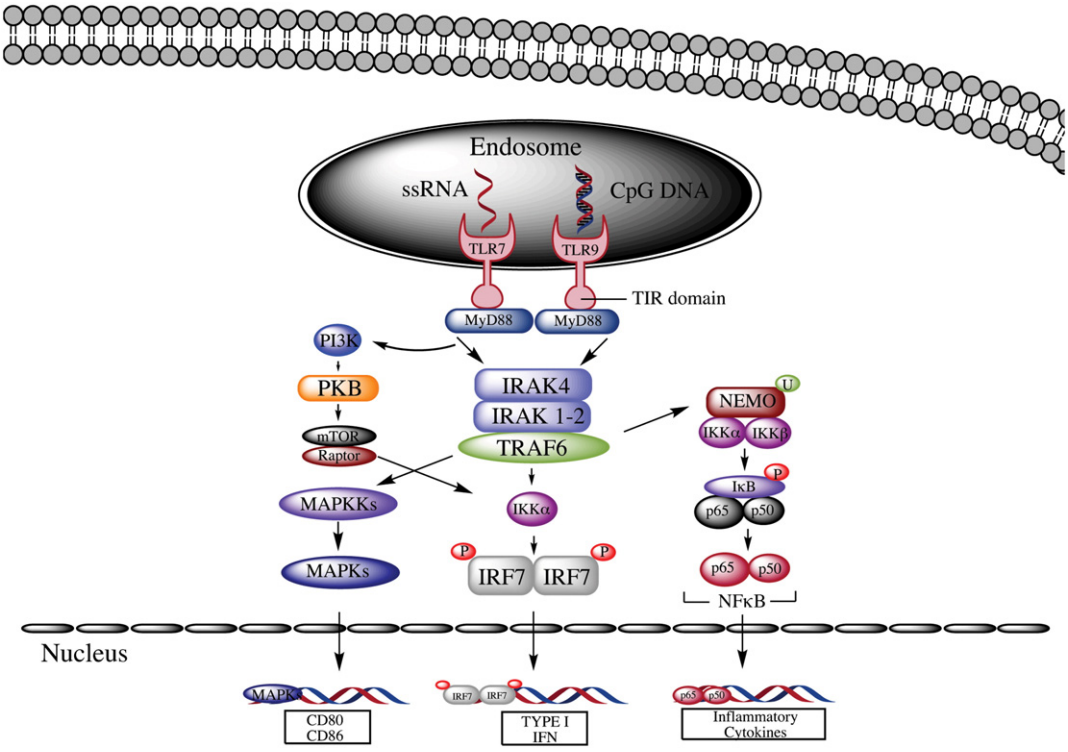

Fig. 1 Activation of endosomal TLR-7 and TLR-9 in pDCs. The activation of TLR-7 or TLR-9 leads to recruitment of the myeloid differentiation primary response protein 88 (MyD88) via its Toll/interleukin-1 (IL-1) receptor (TIR) domain. MyD88 recruits the interleukin-1 receptor-associated kinase 4 (IRAK)-4, which in turn engages IRAK1 or IRAK2. Formation of the resultant complex promotes a conformational change in the kinase domains of the IRAKs, leading to their autophosphorylation and subsequent activation. Activated IRAK kinase complexes phosphorylate the TNF receptor associated factor 6 (TRAF6), which promotes the polyubiquitination of the NF-kappa-B essential modulator (NEMO), as well as itself. Ubiquitinated NEMO then engages the nuclear factor kappa-B kinase subunit alpha and beta (IKKα and β) to form the IκB kinase complex. IκB then liberates NF-κB dimers, leading to their translocation into the nucleus promoding transcription of proinflammatory cytokines. TRAF6 can also activate the mitogen-activated protein kinases kinase (MAPKK), which in turn activates mitogen-activated protein kinases (MAPKs), leading to the upregulation of B7 costimulatory molecules (CD80, CD86). Conversely, TRAF6 can directly interact with IKKα, which phosphorylates IFN regulatory factor 7 (IRF-7), promoting its translocation into the nucleus, thus initiating transcription of type I IFN. Alternatively, MyD88 engagement of phosphatidylinositide 3-kinases (PI3K) followed by protein kinase B (PKB), mammalian target of rapamycin (mTOR), and the adapter protein raptor, activates IKKα and circumvents the IRAK complexes. Activation of TLRs in early endosomes favors expression of IFN whereas activation of TLRs in late endosomes favors expression of inflammatory cytokines.

presence of antigens bound to MHC class II, in conjunction with T cell costimulatory molecules, is mandatory for the priming of naïve T cells in the LNs. Consequently, the ability to upregulate both MHC class II and T cell costimulatory molecules (such as CD40) to prime naïve T cells defines a professional APC. Accordingly, pDCs are clearly APCs, but they are distinctly different from cDCs in their efficiency in priming naïve T cells. Although pDCs have the capacity to prime naïve T cells, cDCs are far more efficient in doing so [62,63]. Additionally, while cDCs and pDCs efficiently migrate to sites of infection, pDCs are less proficient in making their way to the draining LNs [35,64]. Moreover, the few pDCs that do make their way to the draining LNs appear to do so only at the late stages of infection [65]. This observation suggests that pDCs may have a more specific role of antigen presentation at local sites of infection, where a continual supply of antigen is available to populate newly synthesized MHC II. The constitutive turnover of MHC II also suggests that pDCs may be more efficient at presenting endogenous antigens, which may be more available to newly synthesized MHC class II. This idea is consistent with previous reports that pDCs can induce tolerance through the induction of regulatory T cells (Tregs) [66–68].

4.2. Cross-presentation of antigens

Two characteristics that make cDCs exquisitely efficient at activating T cells are first, their ability to take up a wide variety of exogenous antigens from the extracellular environment and load the processed peptides on MHC class II and second, their ability to present exogenous antigens loaded on MHC class I to cytotoxic CD8 T cells (cross-presentation) [69]. The ability of pDCs to perform these functions is less firmly established. So how close are pDCs to cDCs in this capacity? Bastos-Amador et al. recently reported that human pDCs have the ability to capture and present exosomes and apoptotic bodies from Jurkat T cells [70]. These observations are consistent with previous reports that identify pDCs as

phagocytic antigen-presenting cells essential for tolerance to vascularized cardiac allografts in an animal model of organ transplantation [71]. Additionally, immature pDCs are reported to have the capacity to phagocytose and present viral-derived antigens, resulting in naïve $CD8^+$ T cell priming. Hoeffel and colleagues reported that purified human pDCs could cross-present vaccinal lipopeptides and HIV-1 antigens from apoptotic cell to specific $CD8^+$ T cells [72]. Also, in a set of elegantly designed experiments, Tel et al. showed that freshly isolated pDCs can cross-present exogenous antigens to $CD8^+$ T cells, although at a lower capacity than cDCs [73]. Previous studies have shown that pDCs have the capacity to take up antibody-coupled antigens via their C-type lectin DEC-205 receptor, their DC immunoreceptor, and their BDCA-2 receptor [38,74,75]. In a separate report, Tel et al. also showed that pDCs efficiently cross-present antigens to both $CD4^+$ and $CD8^+$ T cells when directed to these same receptors [76]. Additionally, Sandgren and colleagues reported that pDCs efficiently capture recombinant HIV-1 Env protein for antigen presentation via their CD4 receptor [77]. Taken together, these data imply that receptor specificity may be requisite for the ability of pDCs to internalize and (cross-) present a discrete class of exogenous antigens.

5. pDCs as a component of the gut-associated lymphoid tissue

5.1. Localization of pDCs in the gut

The gut-associated lymphoid tissue (GALT) represents the largest immune compartment in the body. In fact, it has been estimated that more than 60% of all T-cells may reside within the small intestine [78], emphasizing the potential contribution of the gut to systemic immunity. Although the role of T and B cells in gut immunity and mechanisms by which they inhabit the gut is well described, an understanding of the immunology and contribution by pDCs is incomplete at best. Due to limitations of access to biological materials, most of what we know about pDC intestinal biology comes from studies conducted using animal models. However, within certain limitations, observations made using animal models are largely consistent with those in humans.

As stated in Section 2.2, pDCs are clearly defined by their unique expression of the C-type lectin receptor CD303 in humans, whereas murine pDCs uniquely express the mouse plasmacytoid dendritic cell antigen-1 (mPDCA-1) [27]. Although the surface phenotype of peripheral pDCs and those residing in the lymph nodes have been extensively characterized [36], pDCs associated with other tissues are less well described. In the steady-state, pDCs are rare in most tissues such as the skin, except at sites of infection or inflammation [79,80], but are relatively abundant in the intestine where they comprise up to 1% of total cells in the epithelium (intraepithelial, IE) and the lamina propria (LP) [31]. The gut microbiota plays an important role in maintaining intestinal homeostasis; however, it is common for pathogenic organisms to infect the intestinal tract and it is therefore important for the immune system to discriminate commensal microbes from pathogenic ones. It has been suggested that tissue-resident DCs in the small intestine are trained by epithelial cells to be tolerogenic to commensal microbes. In contrast, a pathogen assault on the epithelial cells will promote the infiltration of pro-inflammatory DCs and elicit an immune response [81]. pDCs located in the Peyer's patch comprise a unique subset that differs from pDCs located in other tissues, as they fail to secrete type I IFN when stimulated with CpG DNA [82]. It is believed that the local microenvironment of the Peyer's patch promotes gut-pDC anergy and down-regulates their ability to produce type I IFN when stimulated with TLR agonists. Although these cells fail to produce IFN-α when stimulated with TLR agonist, they do produce proinflammatory cytokines as well as stimulate Th17 cell development [83]. The ability of pDCs to play immunogenic or tolerogenic roles has also been shown in other mucosal tissue, such as the lungs where Lombardi et al. demonstrated that murine $CD8\alpha^-\beta^-$ pDCs are immunogenic; however, $CD8\alpha^+\beta^-$ and $CD8\alpha^+\beta^+$ pDCs exhibited tolerogenic properties and effectively supported the conversion of naïve $CD4^+$ T cells into $Foxp3^+$ Tregs [84]. The human equivalent of the murine $CD8\alpha^+\beta^-$ and $CD8\alpha^+\beta^+$ pDCs has yet to be identified as human pDCs do not express the CD8 receptor [36].

5.2. Trafficking to the gut

The steady-state migration of pDCs from the periphery to the intestine relies upon the expression of discrete homing receptors. In contrast to intestinal T and B cells, which express integrin CD103, intestinal pDCs typically do not, nor do they express CCR7 (CD197), which is commonly observed on lymphocytes found in peripheral lymphoid organs (Table 1). Instead, intestinal pDCs express significant levels of β2-integrin (CD18) and moderate levels of $\alpha4\beta7$ integrin (LPAM-1) [31]. Additionally, about half of gut-associated pDCs express P-selectin (CD62P). Also, the majority of intestinal pDCs in the steady-state express the chemokine receptor CCR9 (CD199). This receptor likely plays a critical role in the migration of pDCs under inflammatory conditions as well. Wendland and co-workers reported that more than 95% of pDCs isolated from murine IE and LP express this receptor; however, it is relatively rare on pDCs in the periphery. Consistent with a putative role for CCR9 in the steady-state migration of pDCs to the gut, CCR9 knockout mice show a virtual absence of pDCs in the small intestine, though adoptive transfer of pDCs from wild type mice into CCR9 knockout mice restores normal migration to the small intestine [31]. Mizuno and colleagues reported that $CCR9^+$ pDCs reside preferentially in the small intestine, but not in the colon [85]. They additionally reported that $CCR9^+$ pDCs play a role of central importance in immunological homeostasis of the small intestine in that deficiency of $CCR9^+$ pDCs induced exacerbation of ileitis.

6. Gut associated pDCs and adaptive immunity

6.1. T cell independent activation of B cells by pDCs and gut homeostasis

In humans, IgA is the most abundant antibody isotype found in mucosal secretions and DCs play an important role in promoting their expression [86]. The process whereby B cells acquire the expression of IgA is called "class switching recombination" and occurs via both T-cell-dependent (TD) and T-cell-independent (TI) pathways [87–89]. The IgAs

produced via the TD pathways are of high-affinity and are effective in neutralizing pathogens and their toxins. In contrast, the IgAs produced via the TI pathways are of low-affinity and presumably limit the adhesion of commensal bacteria to intestinal epithelia without neutralizing them [90]. Although both pathways can be activated through cDCs interactions, Tezuka and coworkers reported that pDCs contribute to the TI production of IgA by B cells of the GALT, to a greater extent than cDCs [91]. They also reported that gut-associated pDCs express significant amounts of a membrane bound form of tumor necrosis factor ligand superfamily member 13 (TNFSF13 or CD256) and the B-cell activating factor (BAFF or CD257). Additionally, they demonstrated that the expression of these ligands was upregulated by stromal cell-derived type I IFN and that the expression was required for IgA induction. Finally, they showed that in the mesenteric lymph nodes, stromal cells exclusively expressed type I IFN in the steady state as a result of TLR-dependent recognition of commensal bacteria. In that stromal cell derived type I IFN upregulates CD256 and CD257 and these ligands are necessary for TI production of IgA, these data suggests that functional pDCs and low-level expression of type I IFN by stromal cells in response to commensal bacteria are important for maintaining gut homeostasis.

6.2. pDCs in oral tolerance

Oral tolerance is a form of acquired tolerance that results in immune suppression of T cell-mediated delayed-type hypersensitivity (DHT) reactions to ingested dietary or other non-pathogenic environmental antigens [92]. Although the immune system encounters a myriad of digested antigens and gut-related bacteria, they rarely invoke an inflammatory response. However, in light of the constant potential for orally ingested and bacterial antigen exposure, maintaining proper control of oral tolerance is critical for gut integrity. As an example, when dysregulated, the resultant inflammatory response to dietary antigens or gut microbiota is associated with celiac disease and inflammatory bowel disease, respectively [93–96].

The gut is replete with DCs, particularly in the mesenteric lymph nodes and the liver as well as in the lamina propria and the Peyer's patches of the gastrointestinal (GI) tract [97–100]. The unique environment of the GI tract allows DCs to interact readily with antigens in intestinal lumen or antigens that have transcytosed across intestinal epithelia. Indeed, Worbs et al. reported that orally administered antigens are exclusively recognized by the intestinal immune system and primarily in the mesenteric lymph nodes [101]. In support of this assertion, they observed complete loss of oral tolerance in mice following mesenteric lymphadenectomy. For these reasons, a fundamental role for DCs in oral tolerance is not unexpected (reviewed by Rescigno [102]). Multiple mechanisms have been described in relation to the induction of oral tolerance; for example, the activation of antigen-specific Tregs is primarily attributed to $CD103^+$ cDC in the lamina propria of mice, where these cells express the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO) and have been shown to promote $Foxp3^+$ Treg development via a TGF-β and retinoic acid (RA)-dependent mechanism [103,104]. However, in humans, the expansion of tolergenic DCs, which drive Treg development, is additionally dependent on presence of thymic stromal lymphopoietin [105]. Also, low-dose antigen feeding has been reported to promote the expansion of $CD4^+CD25^+$ Tregs [106,107] and Th3 cells [108], which, upon adoptive transfer, can suppress inflammatory responses [109].

Oral tolerance can also be promoted through a mechanism that results in the depletion of antigen-specific T cells or though the inactivation of reactive T cells (anergy). Recent evidence suggests that cytotoxic $CD8^+$ T cells are important contributors to diseases characterized by severe chronic GI inflammation and dysregulation of tolerance such as celiac disease and inflammatory bowel disease (IBD) [110,111]. Using a model of allergic contact dermatitis and a classical model of DTH, mediated by specific $CD4^+$ T cells to ovalbumin, Goubier et al. observed that antigen feeding prior to skin sensitization induced tolerance and inhibited $CD8^+$ T cell priming and additionally prevented the development of delayed-type contact hypersensitivity by a mechanism that involved both Tregs and pDCs [112]. In a subsequent report, Dubois and coworkers demonstrated that pDCs promoted the deletion of Ag-specific $CD8^+$ T cells in the GALT and that Treg inhibition of residual effector T cell priming is both sequential and complementary events that are mandatory for complete ablation of systemic $CD8^+$ T cell-mediated DTH responses to orally administered antigens [113].

7. Gut-associated pDCs and human disease

7.1. Inflammatory bowel disease and other autoimmune diseases

Over the past decade, it has been suggested that pDCs play a role in the pathogenesis of autoimmune irritable bowel diseases (IBDs) including Crohn's disease, and ulcerative colitis (UC), although a consensus on this subject has not been reached. Baumgart and coworkers observed an increase in pDC numbers in the inflamed colonic mucosa and mesenteric lymph nodes of subjects with IBD [114]. They additionally reported that increased gut mucosa pDCs were associated with a decrease in pDCs in the periphery of these subjects [115] and that the decrease of pDCs in the periphery directly correlated with the severity of disease [115]. Interestingly, they also reported that pDCs in the periphery of those with IBD upregulate the $\alpha4\beta7$ integrin receptor, which as stated above, is associated with migration to the gut and with a mature phenotype. Additionally, a higher percentage of circulating pDCs were reported to express CD40 and CD86 in cases with IBD as compared to healthy controls [114,115]. Lastly, they observed that expression of TNFα, IL6, and CXCL8 was significantly higher in pDCs from cases with IBD compared to controls [114]. In contrast, Middel and colleagues reported that they observed no differences in pDC numbers in colonic tissue from Crohn's disease cases and controls [116]; and similar observations were reported by Mannon et al. [117]. Further studies will be necessary to resolve these discrepancies; however, these data raise the possibility of pDC involvement in autoimmune gut pathology. We have recently reported an increased infiltration of pDCs in the duodenum of cases with symptoms consistent with myalgic encephalomyelitis [118], a disease that often presents as a comorbid condition of irritable

bowel syndrome [119]. The increase in duodenum pDCs may be the result of GI inflammation or infection; however, additional studies are currently being conducted to address this observation.

Associations with pDCs and several other autoimmune diseases have been reported including multiple sclerosis (MS), psoriasis, type 1 diabetes, Sjögren's syndrome, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [120–125]. For example, in most cases of SLE, an over-expression of type I IFN is observed which correlates with disease activity and severity [126]. Lande and coworkers identified immunogenic complexes composed of neutrophil-derived antimicrobial peptides and self-DNA in the sera of SLE cases [125]. They further reported that these complexes activate pDCs via TLR-9 leading to the upregulation of type I IFN and the development of autoantibodies. Associations of autoimmunity and the dysregulation of type I IFN expression have prompted researchers to target pDCs for the treatment of diseases such as SLE and RA. Barrat and coworkers observed a significant reduction in autoantibodies, a decrease in end-organ damage and increased survival in lupus-prone mice treated with the specific TLR-7 and 9 inhibitor IRS 954 [127]. In another study, Laio et al. observed that the TLR-9 inhibitor RO9021 suppressed type I IFN expression as well as the progression of arthritis in a mouse model of collagen-induced arthritis [128]. In contrast to SLE, subjects with MS are often reported to have a suboptimal IFN response [129] and many of those individuals have been effectively treated with type I IFN since the early 1980's [130].

Although a comprehensive review of pDCs and autoimmunity is beyond the scope of this review, we would refer the reader to the recent publication of Ganguly et al. [131] for a comprehensive review of pDCs and their role in autoimmunity.

7.2. Redistribution of pDCs to the gut in HIV/AIDS cases

Previous studies have demonstrated that the GI tract is preferentially and significantly affected during the course of HIV infection (reviewed by Brenchley and Douek [132]). Indeed, histological abnormalities in gut tissue of HIV cases were first described by Kolter and colleagues in 1984 [133] and subsequent studies have revealed a decreased regenerative capacity of the gut epithelium and increased mucosal permeability in HIV cases [134–136]. Additionally, depletion of $CD4^+$ T cells is found to be a characteristic marker of HIV infection [137] and is only partially restored by highly active antiretroviral therapy (HAART) [138–141]. Importantly, studies using rhesus macaques have confirmed that the GALT is the principle site of HIV replication and occurs early in the course of infection [142].

pDCs express the CD4 receptor, as well as the CCR5 and CXCR4 co-receptors, making them susceptible to HIV. Their expression also suggests why pDCs are more susceptible than cDCs to HIV [143,144]. HIV is typically delivered to pDCs by $CD4^+$ T cells and activation of the pDC by CD40 ligand (also delivered by the $CD4^+$ T cell), promotes viral replication [143]. Infected, pDCs may potentially play a role in viral dissemination since they readily transmit HIV to $CD4^+$ T cells in vitro, and such transmission is also augmented by CD40 ligand activation [145,146].

Studies of the pDC numbers in the blood of HIV cases demonstrate a progressive depletion of these cells, which correlates with plasma viral load [146–150]. Similar data were shown in the blood of macaques infected with simian immune deficiency virus (SIV) where the number of pDCs was reduced 3-fold [149]. Remarkably, circulating pDCs were reported to express the gut-homing marker $\alpha4\beta7$, suggesting pDCs were primed for migration into the gut. Subsequent evaluation of the rectal tissue of HIV infected animals revealed a fourfold increase in the number of pDCs as compared to naïve animals. These data support the hypothesis that the loss of pDCs in the periphery was a result of their redistribution into the gut. In further support of this, Li and colleagues have demonstrated a fourfold increase in pDC accumulation in jejunum, colon, and gut-draining LNs of SIV infected rhesus macaques [151]. At the same time there was no pDC accumulation in the liver and peripheral LNs. Similar distributions of pDCs have been observed in HIV cases. For example, increased pDC accumulation was detected in the terminal ileum of HIV infected cases by Lehmann and colleagues [152]. Tissue homing was explained by significant upregulation of the gut-homing receptor CD103 as compared to uninfected controls. Indeed, HIV infection appears to dictate pDC migration exclusively into the gut and this migration is determined by the expression of gut homing receptors. The mechanism responsible for the upregulation of gut homing receptors in HIV cases has yet to be determined; however, our initial concept of HIV as a disease of CD4 T cells must now be reevaluated, and equally considered a disease of the gut involving the redistribution and dysregulation of pDCs.

8. Concluding remarks

pDCs are relatively recent additions to the field of immunological research; however, their significance is being realized at a prodigious rate. Through the engagement of TLR-7 and TLR-9 by ssRNA and CpG DNA respectively, they produce substantially more type I IFN than any other cell. It is because of these pattern recognition receptors that their involvement in the innate antiviral response was initially considered their principal function, and indeed, their role in pathogen response cannot be disputed. Nevertheless, they are now being appreciated for their involvement in many other areas, including tolerance induction and their contribution to human diseases such as autoimmunity and HIV/AIDS. Most of our current understanding of pDC biology has been conducted using pDCs derived from whole blood or from the spleen of mice. As a result, the biology of gut-associated pDCs is largely unexplored, but current research suggests that these cells likely play a critical role in many aspects of immunity and many exciting and undiscovered things await our investigation.

Conflict of interest statement

The authors declare no conflicts of interest.

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Plasmacytoid dendritic cells, a role in neoplastic prevention and progression

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Abstract

Plasmacytoid dendritic cells (pDCs) are multifunctional bone marrow derived immune cells that are key players in bridging the innate and adaptive immune systems. Through the engagement of endosomally located toll-like receptors 7 and 9, they produce more type I interferon than any other cell in circulation. Unique among immune cells, pDCs serve two professional roles, one as IFN-producing cells and the other as antigen presenting cells. In light of their robust interferon producing abilities and the mechanism by which they are activated, their principal function was long thought to be limited to antiviral responses; however, it is now understood that pDCs serve other immune roles such and the induction of oral tolerance. Current research suggests that pDCs have cytotoxic potential and can effectively induce apoptosis of tumor-derived cells lines. They are also reported to display tolerogenic function with the ability to suppress T cell proliferation, analogous to regulatory T cells. In this capacity, they are critical in the suppression of autoimmunity but can be exploited by tumor cells to circumvent the expansion of tumor-specific T cells, thereby allowing tumors to persist. In this mini-review we will explore the fascinating contribution of pDCs to neoplastic pathology and discuss their potential utilization in cancer immunotherapy.

Introduction

Plasmacytoid dendritic cells (pDCs) are a unique population of bone-marrow-derived immune cells that bridge the innate and adaptive immune systems. They are remarkable in that they are the only immune cell to serve two professional roles, one as interferon (IFN)-producing cells and the other as antigen presenting cells (APCs). Although accounting for only 0.3 to 0.5% of peripheral blood cells, pDCs are responsible for over 95% of type I IFN produced by circulating lymphocytes [1]. Activation of pDCs and the subsequent production

of IFN occur as the result of a signaling cascade that initiates through the receptor-ligand interactions of pattern recognition receptors (reviewed by Lombardi and Khaiboullina [2]). pDCs are primarily activated through the engagement of endosomally located toll-like receptors (TLR)-7 and TLR-9, by ssRNA [3, 4] or non-methylated and CpG DNA [5, 6], respectively (Figure 1A); which are common to microbial genomes, such as viruses or their replicative intermediates. pDCs are also known to produce type I IFN in response to double stranded dsRNA, likely through the engagement of protein kinase R (PRK) [7], although their response to dsRNA is less well characterized. Similar to other TLRs, (with the notable exception of TLR-3), TLR-7 and TLR-9 utilize the universal adapter protein MyD88 (myeloid differentiation primary response 88), which acts via the constitutively expressed transcription factor IRF7 and the inflammatory transcription factor NF-κB, thereby initiating transcription of type I and III IFN, or inflammatory cytokines and chemokines, respectively [8-10]. Upon activation, pDCs also undergo phenotypic changes resulting in the upregulation of costimulatory molecules, including, CD40, CD80, CD86. They ultimately develop into more "conventional" dendritic cells (cDC) with classical DC morphology and the ability to present and cross-present antigens in the context of MHC and costimulatory molecules to naïve and memory T cells [11].

Over the last decade our understanding of pDCs biology has greatly expanded but this expansion has also resulted in many unanswered questions. Indeed, it is now evident that pDCs play a much larger role in immunology than originally realized. In addition to their ability to produce IFN, pDCs contribute to tolerance but when dysregulated can also contribute to autoimmunity. Current research suggests that pDCs have the capacity to

induce apoptosis of neoplastic cells and, therefore, may also contribute to cancer surveillance. Conversely, it has also been shown that the tolerogenic functions of pDCs may be utilized by tumors to their advantage, allowing them a way to evade the immune system. For these reasons, an understanding of pDC function in the context of neoplastic pathology and the tumor microenvironment will likely provide a greater understanding of malignancy in general and suggest potential treatment strategies.

Neoplastic progression and the tumor microenvironment

The historical paradigm of cancer development and propagation is based upon the presence of mutations that lead to cell cycle dysregulation. According to this model, a single mutation in a cell cycle gene allows the cell to grow uncontrolled whereby it rapidly expands to form a tumor. However, this model is an oversimplification in that as the tumor expands, it forms its own microenvironment that differs from that of healthy non-malignant tissue [12]. Indeed, crosstalk between stromal and epithelial cells is essential for maintaining homeostasis of malignant as well as nonmalignant tissue [13, 14]. In the last three decades, our knowledge regarding the role of immune effector cells in maintaining a protumorigenic microenvironment has increased substantially. For instance, tumor derived colony-stimulating factor-1 (CSF-1), VEGF and endothelial monocyte activating polypeptide II (EMARII) have been shown to facilitate the infiltration of tumor tissue by monocytes [15-17]. Within the tumor, monocyte derived macrophages polarize into the M2 stage, which is strongly associated with proangiogenic and protumorogenic properties [18, 19]. Furthermore, tumor associated macrophages contribute to an immunosuppressive environment by releasing interleukin (IL)-10 and TGF-β [20] and additionally, promote the infiltration of T regulatory cells (Tregs) by releasing the chemoattractant CCL22 [21, 22]. A positive correlation has been observed between inducible costimulator (ICOS)-expressing

Tregs and pDCs in the peripheral blood and peritumor tissue of subjects with gastric cancer [23] (Figure 1B). Additionally, ICOS-driven interaction between CD4+ T cells and pDCs has been reported to lead to the upregulatyion of Tregs and IL-10 secretion in breast tumors [24]. These observations underscore the contributions of immune effector cells to an immunosuppressive tumor microenvironment, thus supporting the maintenance and propagation of malignancy.

Plasmacytoid dendritic cells, immunity, and cancer

All DCs are professional APCs with the capacity to prime and activate naïve T lymphocytes [25]. By controlling the outcome of antigen presentation to T cells, DCs also play a central role in the maintenance of peripheral tolerance. Through the activation of pattern recognition receptors, such as TLRs, they also produce cytokines such as interferons and interleukins, thus modulating the balance between humoral immunity, cell-mediated immunity and tolerance [26]. For these reasons, it is not surprising that DCs may play a pivotal role in antitumor immunity. Involvement of pDCs in neoplastic disorders became evident upon the observation that several tumors including ovarian, head and neck, and breast tumors and primary melanoma are infiltrated with pDCs [27-32]. In some instances, the presence of infiltrating pDCs is associated with a poor prognosis; for example, while investigating epithelial ovarian cancer (EOC), Conrad and coworkers observed that a significant number of Foxp3+ Tregs present in the tumor microenvironment expressed ICOS [33]. They further observed that the ability of these cells to suppress T cell proliferation was strictly dependent on ICOS-L costimulation provided by infiltrating pDCs and therefore suggested that pDCs and ICOS+ Foxp3+ Tregs were strong predictors of EOC progression. As a further example, Aspord et al. reported that Th2-promoting pDCs were associated with the progression of melanoma and that the frequency of IL-5, 10, and 13-

producing T cells in melanoma cases was correlated with a high proportions of OX40L- and ICOSL-expressing pDCs [34].

Dendritic cells play a central role in orchestrating immune responses and numerous studies have reported that tumor tissue is often infiltrated with various populations of DCs including pDCs. For example, as previously stated, pDCs have been reported to be among the cellular infiltrate of several tumors [27-32]. It is believed that the recruitment of pDCs into tumor tissue is governed by chemokines secreted by neoplastic cells. Zou et al., as well as others, have reported that tumors infiltrated with pDCs express high levels of chemokines such as CXCL12 (stromal cell-derived factor 1) and CCL20 (macrophage inflammatory protein-3) [29, 32, 35]. Zou and coworkers additionally reported that tumor-derived pDCs express high levels of CXCR4 [29], the specific receptor for CXCL12 [36, 37]. Charles et al. reported that tumor-associated pDCs express high levels of the chemokine receptor CCR6, the receptor for CCL20 [35], a requirement for the rapid recruitment of dendritic cells into tissue [38]. Indeed, the multiple receptor-ligand interactions that occur between tumor cells and immune effector cells contribute to the complex microenvironment that allows tumors to maintain their own persistence.

pDCs are essential for recognition of altered self-antigens and for triggering immune responses directed toward transformed cell. Therefore, it would be expected that the increased presence of pDCs in tumor tissue should promote immune recognition of tumor antigens and, in turn, lead to tumor rejection. Contrary to this supposition and unexpectedly, increased pDC tumor infiltration is often associated with tumor progression and persistence [24, 39]. Furthermore, it has been shown that increased pDC infiltration is associated with poor prognosis in some cancer cases [40, 41]. Therefore, it has been

suggested that tumor-associated pDCs are often incompetent with respect to tumor-specific immune surveillance.

Several mechanisms have been suggested to explain tumor-associated pDC dysfunction including the recruitment of immature pDCs, promotion of pDC tolerance and tumor secretion of immunosuppressive factors. Numerous studies have shown that tumor-associated pDCs are immature as characterized by the lack of expression of costimulatory molecules such as CD80, CD83 and CD86 [32, 42, 43]. In addition to being immature, these pDC are shown to be defective in IFNα production [32, 44] and it has been suggested that defective IFNα production is the result of a downregulation of Flt3, TLR9 or IRF7 [44-47]. Tsukamoto et al. [48] proposed that tumor-associated immunoglobulin-like transcript 7 ligands (ILT7L) can downregulate IFNα production in pDCs via interaction with the ILT7 receptor. IFNα is a pleotropic cytokine with strong tumor inhibitory activity [49]. Therefore, by producing less IFNα, pDCs may significantly impair local immune surveillance allowing tumors to escape IFNα-associated immune responses. Several studies have suggested that tumor-associated pDCs are indeed tolerogenic [28, 50]. For example, animal tumor models have shown that tumor-infiltrating pDCs can activate mature Tregs [51, 52]. Additionally, it is well documented that malignant cells and tumor associated-pDCs release indoleamine 2,3-dioxygenase (IDO) which is a powerful promoter of Treg activation, and can lead to anergy, thus allowing tumor cell to escape immune surveillance [50, 53, 54].

Although pDC infiltration of tumors is often associated with disease progression, their activation with TLR-agonists is proving to be an effective treatment for some forms of neoplasm. For instance, the topical treatment of basal cell carcinoma, superficial squamous cell carcinoma and some superficial malignant melanomas, with the synthetic TLR-7 agonist

Imiquimod, has been shown to lead to the increased infiltration of activated pDCs and a significant reduction in neoplastic cells and in some cases, a complete regression [31, 55-58].

Anti-neoplastic functions of pDCs

DCs have the potential to invoke antitumor immunity in multiple ways. Similar to cytotoxic CD8 T-lymphocytes (CTLs), natural-killer (NK) cells and gamma/delta T cells; DCs have the capacity for direct cytotoxic killing of susceptible target cells such as virus infected cells and transformed cells. The focus or our discussion is the contribution of pDCs to anticancer immunity; notwithstanding, significant body of research also addresses the cytotoxic capacity of cDCs. These topics are excellently reviewed by Tel et al., and Larmonier et al., with respect to humans and animal models [59, 60] and thus, will only discuss the use of animal models as it pertains to human disease.

Classic cytotoxic cells, such as NK cells and CTLs, express perforin (PRF1) and the proapototic enzyme granzyme B (GZMB). Although initially believed that PRF1 was required for entry of GZMB into target cells, current research suggest that both proteins may enter cells through an alternative mechanism. For instance, Veugelers and colleagues proposed a mannose 6-phosphate receptor as a potential entry mechanism for PRF1 and GRZB [61]. The definitive role for pDC-GRZB is currently the subject of ongoing investigations. However, since pDCs do not express PRF1, an alternative PRF1-independent entry method would support the possibility of pDC cytotoxicity in a GZMB-dependent but PRF1-independent manner. Indeed, using a human asthma model of segmental allergen challenge, Bratke and coworkers reported that pDCs upregulate GRZB in response to IL-3 and additionally showed that IL-3 activated pDCs killed MHC deficient K562 cells [62].

Furthermore, they reported that the observed killing was abrogated in the presence of GRZB and caspase inhibitors. Interestingly, they also observed that engagement of the TLR-7 or -9 receptor suppressed GRZB expression, suggesting that the classical IFN-induced pathway of pDCs is not involved in GRZB-associated cytotoxicity. Tel et al. reported that human pDCs activated with the preventative vaccine to tick-borne encephalitis virus FSME upregulated the neural cell adhesion marker CD56, a classic NK marker, and were empowered with the tumoricidal ability to lyse K562 and Daudi cells in a contact-dependent manner [63]. They additionally reported that the expression of CD56 on the surface of pDCs coincided with elevated expression of programmed death-ligand 1 (PD-L1), GRZB and TNF-related apoptosis-inducing ligand (TRAIL).

TRAIL-dependent apoptosis has been implicated in the tumoricidal capacity of pDCs by other researchers as well. For instance, Stary and coworkers reported that, upon treatment of basal cell carcinoma with Imiquimod, a cellular infiltrate of GRZB and PRF1 positive cDCs and TRAIL positive pDCs was observed [64]. However, in contrast to the observations of Bratke et al., the contribution of pDC killing was strictly TRAIL dependent, as TRAIL neutralizing antibody abrogated the killing of TRAIL-sensitive Jurket cells. Consistent with the observations of Stary et al., Kalb and coworkers reported that pDCs stimulated with agonists for TLR-7 and 9, but not other TLRs, upregulated the surface expression of TRAIL in a type I IFN-dependent manner [65]. They additionally reported that pDCs treated with TLR7/9 agonists as well as pDCs treated with type I IFN efficiently lysed Jurkat cells, as well as the melanoma cell lines SKMel2 and WM793, in a TRAIL and contact-dependent manner. Using a mouse model of melanoma, Drobits and coworkers showed that topical Imiquimod treatment resulted in tumor clearance in a TLR7/MyD88-dependent and IFN-α/β receptor 1-dependent manner, with a concomitant upregulation of the

chemokine CCL2 in mast cells [66]. They additionally observed that Imiquimod treatment promoted the secretion of both TRAIL and GRZB and that blocking these molecules led to impaired pDC-mediated tumor killing. These data strongly implicate both TRAIL and GRZB in pDC-mediated tumor killing and further suggest that the tumoricidal ability of pDCs is independent of adaptive immunity.

pDCs as potential targets in cancer immunotherapy

CTLs are considered to be the most critical mediators of anticancer immune responses and CTL infiltration of tumors is typically associated with a positive diagnostic outcome [67, 68]. The use of immunomodulating drugs to increase CTL responses has been shown to be an effective strategy to improving the induction of long-term memory CTLs. For instance, one strategy targets the blockade of the inhibitory receptors such as the cytotoxic T lymphocyte–associated antigen 4, the programmed death-1 receptor (PD-1) or its ligand, PD-L1. This approach is often referred to as "immune-checkpoint blockade." The use of anti-PD1 antibodies in combination with the anti-B cell drug rituximab has led to encouraging results both in preclinical models and in clinical applications [69, 70]. However, the non-specific nature of these "check point inhibiting" drugs and the broad mechanism by which they exert their actions can lead to activation of autoreactive T cells and, in turn, lead to potentially severe side effects [71, 72]. DC therapies are an attractive alternative to check point-inhibiting drugs in that they have few side effects and natural DC therapy is generally less costly.

Ex vivo DCs are capable of inducing CTL responses against tumors when loaded with tumor-associated antigens and given as a vaccine. Therefore, the primary goal of cancer vaccine immunotherapy is the induction of long-term memory CTLs that are capable

of facilitating immune surveillance and promoting tumor rejection. Although the use of cancer vaccines to generate antitumor immune responses is theoretically promising and appears fairly straightforward, the clinical success of such vaccines have been less than encouraging [73]. Although previous studies have largely employed monocyte derived DCs (moDCs) for this purpose, a pioneering study conducted in the laboratory of Dr. Jolanda De Vries, utilized activated pDCs pre-loaded with tumor associated antigens to vaccinate subjects with melanoma [74]. Although the overall magnitude of antimelanoma immune responses was comparable to that of previous moDC trials, a number of encouraging observations were made as a result of this study. The pDC vaccine produced a systemic type I IFN response, which is critical to NK activation and subsequent inhibition of tumor metastasis [75]. Additionally, pDCs were observed to migrate efficiently to the lymph nodes and, subsequently, T cell clones with high avidity could be identified after vaccination, indicative of a strong functional response. Lastly and most importantly, the overall survival of subjects treated with the vaccine greatly increased when compared to matched controls that only received a standard chemotherapy treatment. With regarding the mechanism of the observed efficacy, one could speculate that the improved treatment outcome may have been the result of pDC-mediated activation of innate immune cells such as NK cells or perhaps T cells induced by pDCs may be more potent immune effectors. Nevertheless, these observations clearly suggest that pDC-based anticancer vaccines will likely provide advantages over moDC vaccinations or may even supplement moDC vaccinations when used in combination therapy.

Concluding remarks

The current model of tumor neogenesis holds that the tumor microenvironment provides favorable conditions that support malignant cell growth and propagation, while at the same

time, allows them to evade the immune system. pDCs that infiltrate tumors are often dysfunctional and, accordingly, do not produce IFNα. Furthermore, they often display an immature or naïve phenotype and promote a tolerogenic microenvironment through the activation of Tregs. In this context, pDCs likely contribute to neoplastic homeostasis and, accordingly, represent a very attractive target in cancer immunotherapy. Indeed, activating pDCs with the TLR-7 agonist, Imiquimod is highly effective in treating some forms of skin cancer and exemplifies the potential impact of pDC immunity in neoplastic disease. Additionally, pDCs have been shown to have tumoricidal properties in culture; therefore, potentiating this ability in vivo may prove to be an effective treatment strategy. Future studies regarding the contributions of pDCs to malignancy will likely afford many opportunities for immunotherapy strategies.

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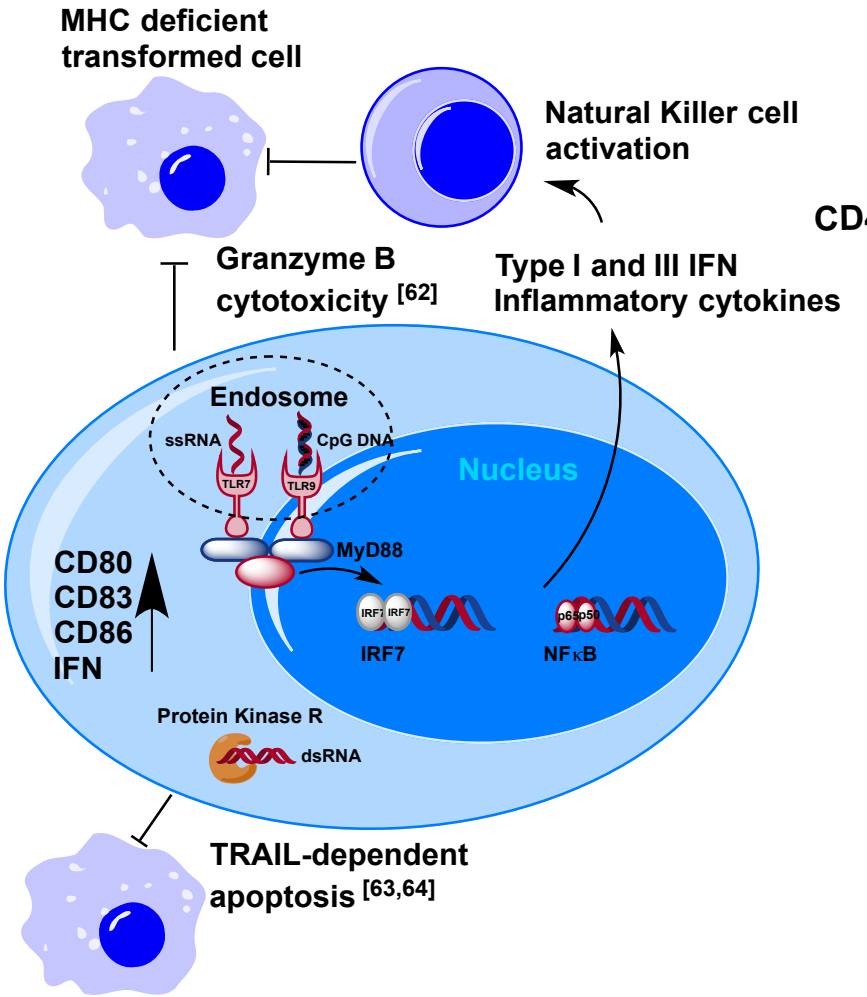
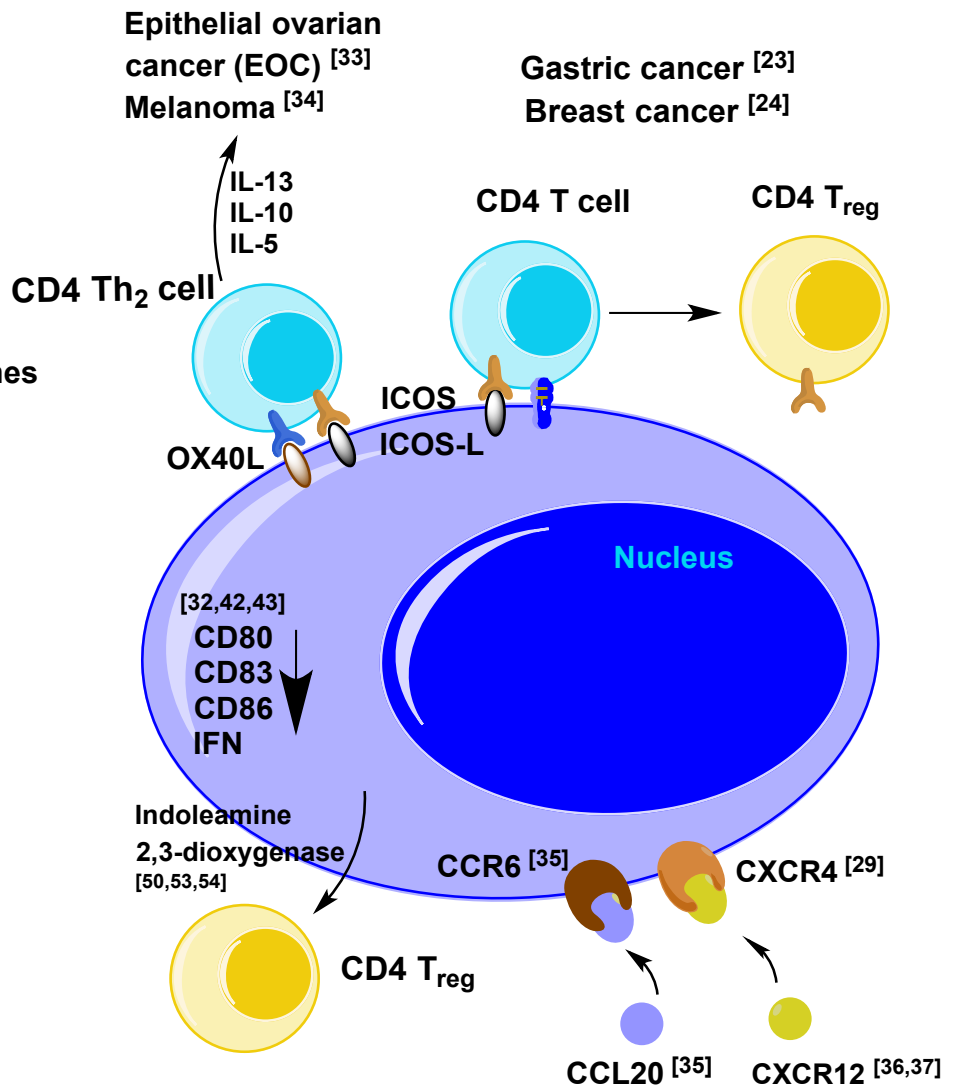
Figure ledged

Figure 1. Plasmacytoid dendritic cell (pDC) involvement in tumor inhibition and promotion. Fig. 1A. Activation of pDCs through the engagement of endosomally located toll-like receptors (TLR) 7 and 9 by ssRNA or non-methylated CpG DNA, respectively, leads to the MyD88 dependent upregulation of type I interferon (IFN) and inflammatory cytokines as well as expression of costimulatory molecules such as CD80, CD83 and CD86. Type I IFN expression also occurs through the engagement of protein kinase R (PKR) by dsRNA. pDCs possess direct tumoricidal activity in a granzyme B and TRAIL dependent manner and indirectly through the activation of natural killer (NK) cells by type I IFN. Fig. 1B. Mechanisms suggested to explain tumor-associated pDC dysfunction include the recruitment of immature pDCs as characterized by the lack of expression of costimulatory molecules, and tumor secretion of immunosuppressive factors. In addition to being immature, these pDC are shown to promote tolerance by activating Tregs, express anti-inflammatory cytokines such as IL-13 and are refractory in IFN production.



Mature pDC
Tumor inhibition

Figure 1A



Immature pDC
Tumor promotion

Figure 1B